

**Signalling pathways mediating
ephrin-A-induced growth cone
collapse.**

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Abstract.

The ephrin-A family of axon guidance cues, which activate the EphA family of receptor tyrosine kinases, guide the axons of many types of neuron to the correct target during embryonic development. One particularly well-studied example is the projection of RGC processes to precise positions in the midbrain target that reflect the position of the RGC in the retina. Ephrin-As are membrane-tethered molecules expressed in a gradient in the midbrain, and they govern the formation of the retinotectal map by differential, contact-mediated repulsion of Eph-A-expressing RGC axons. In order to identify signalling molecules that mediate ephrin-A induced repulsion of RGCs, I have developed a novel co-culture assay in which contact with a single non-neuronal cell that expresses endogenous levels of ephrin-A induces rapid loss of RGC growth cone lamella, followed by axon retraction. I have confirmed that these cellular responses are mediated by neuronal EphA receptor signalling and, in combination with the traditional soluble collapse assay, have used this physiologically relevant co-culture assay to identify a more specific role for the Rho effector ROCK in ephrin-A-induced RGC responses than has previously been published. Specifically ROCK activity mediates ephrin-A-induced RGC axon retraction, but not loss of growth cone lamella. I have also identified the non-receptor tyrosine kinase Abl as having a major role in the ephrin-A-induced RGC repulsive response, as the Abl kinase inhibitor STI571 prevents both the ephrin-A-induced loss of RGC lamella and axon retraction. I have demonstrated the existence of a protein complex containing active Eph receptors, Abl and Mena, and shown that disruption of this complex correlates with STI571-dependent inhibition of the ephrin-A-induced RGC repulsive responses. These results comprise the first evidence that Abl plays a role in mediating Eph receptor signals, and is involved in the cytoskeletal rearrangements that underlie ephrin-A-induced growth cone collapse *in vitro*, and thus both complement and extend the published evidence demonstrating a role for Abl in mediating axon guidance *in vivo*.

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Chapter 1. Introduction.

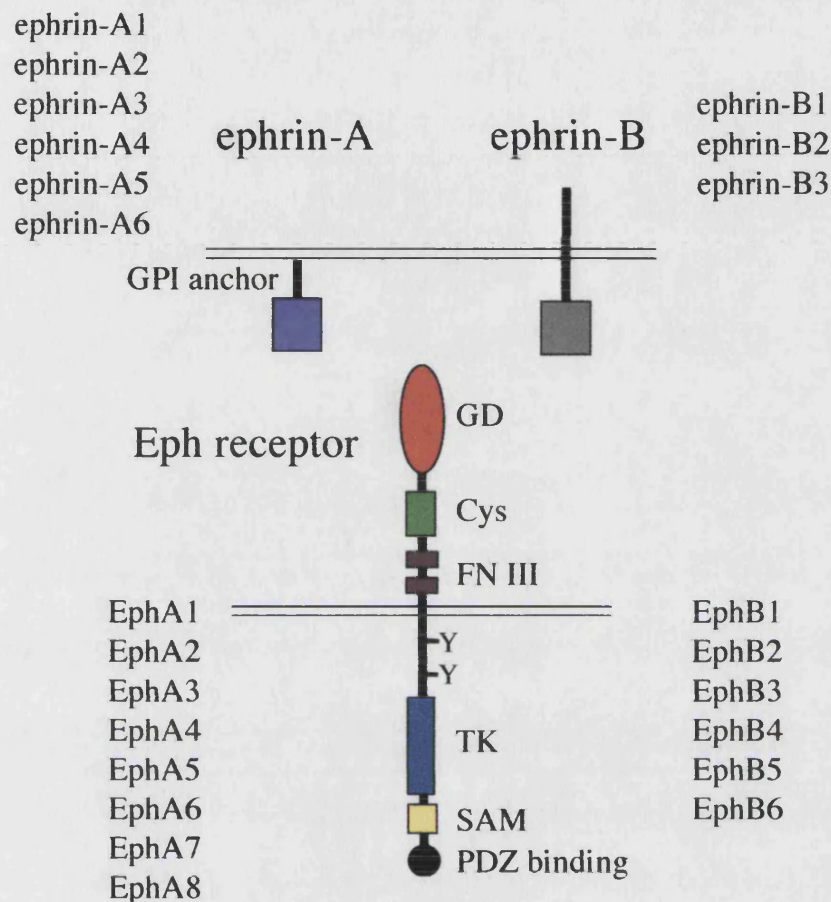
1.1. Introduction.

During development neuronal processes must extend, often over great distances, to reach their correct target and produce a functional adult nervous system. The neuronal growth cone at the leading edge of an extending axon senses extracellular guidance cues, and transduces intracellular signals that ultimately regulate the speed and direction of axon outgrowth in response to these cues. The Eph receptor family of tyrosine kinases direct many critical process during embryogenesis, and they have a particularly well studied role in axon guidance (Kullander and Klein, 2002; Wilkinson, 2001). Co-ordinated changes in cell shape and motility underlie many of the morphogenic events controlled by Eph receptors and this is reflected in the fact that intracellular pathways initiated by Eph receptor activation ultimately converge on the actin cytoskeleton and cellular adhesion machinery (Kullander and Klein, 2002; Murai and Pasquale, 2003; Wilkinson, 2001). However the signalling pathways that mediate cellular responses during Eph receptor dependent axon guidance decisions remain incompletely characterised.

1.2. Eph receptor and ephrin families.

All ligands for the Eph family of receptor tyrosine kinases (RTKs) are membrane-bound molecules, known as ephrins, and this family of proteins is split into two classes, ephrin-As and ephrin-Bs (1997; Gale et al., 1996b). Ephrin-B molecules contain a transmembrane region and a short but highly conserved cytoplasmic tail, whereas ephrin-A molecules are attached to the outer leaflet of the cell membrane via a GPI linkage (Fig 1.1). Eph receptors are similarly divided into two classes based on their sequence similarity and ligand binding affinity. Although there is significant promiscuity in the binding of Eph receptors and ephrins within classes, interactions are restricted such that EphB receptors bind only to ephrin-B ligands and EphA receptors bind ephrin-A ligands. The exception is EphA4, which is known to bind members of both the ephrin-A and ephrin-B classes (Gale et al., 1996b). Eph-ephrin interaction results in phosphorylation of tyrosine residues within the Eph receptor

Fig 1.1. Structure of Eph receptors and ephrins.



Ephrin-As are attached to the membrane via a GPI-linkage, ephrin-Bs are transmembrane proteins. Eph receptors have an extracellular globular domain (GB), a cysteine-rich motif (Cys), and two fibronectin type III motifs (FN III). The intracellular Eph receptor tail contains two highly conserved tyrosine residues in the juxtamembrane region (Y), a central tyrosine kinase domain (TK) a sterile alpha motif domain (SAM) and at the C-terminus there is a PDZ-domain-binding motif.

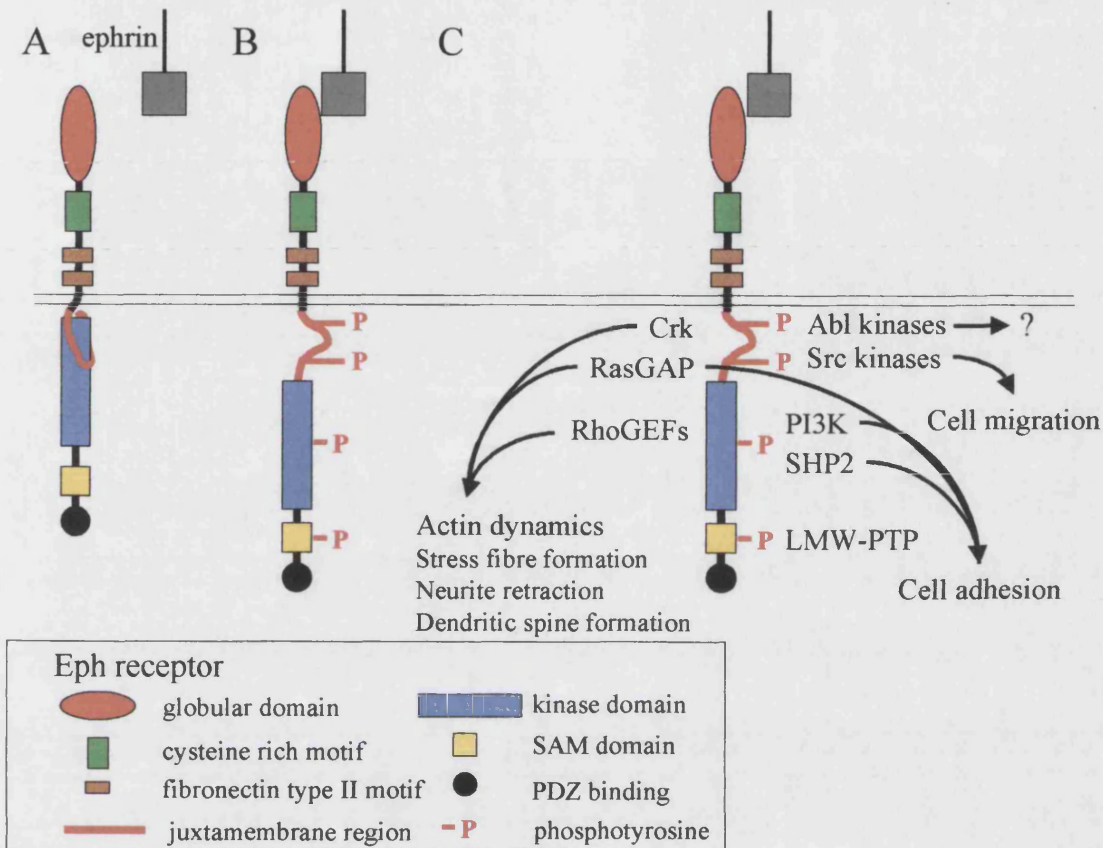
cytoplasmic domain (Davis et al., 1994). Eph receptor phosphorylation is required for Eph kinase activity and the recruitment of SH2- (Src homology 2) containing molecules, and both are thought to contribute to the induction of Eph receptor dependent signalling cascades (Binns et al., 2000; Ellis et al., 1996; Holland et al., 1997; Yu et al., 2001; Zisch et al., 1998; Zisch et al., 2000). Interestingly ephrin-Bs also become phosphorylated following Eph receptor binding, and there is increasing evidence that signals can also be transduced by the ephrin-expressing cell (Cowan and Henkemeyer, 2002; Murai and Pasquale, 2003).

1.2.1. Eph receptor structure and binding partners.

Like all known RTKs, Eph receptors are type 1 transmembrane proteins (Fig 1.1). They consist of a N-terminal globular domain that is both necessary and sufficient for ligand binding followed by a cysteine-rich domain and two fibronectin type III repeats, which might be involved in receptor clustering (Himanen et al., 1998; Labrador et al., 1997; Lackmann et al., 1998). Just inside the membrane is a pair of tyrosine residues that are highly conserved throughout the Eph receptor family (Fox et al., 1995; Sajjadi and Pasquale, 1993). These juxtamembrane tyrosines are major autophosphorylation sites, and Eph receptors are phosphorylated on these residues *in vivo* at sites of potential Eph receptor-ephrin interaction. (Choi and Park, 1999; Ellis et al., 1996; Kalo et al., 2001; Kalo and Pasquale, 1999; Zisch et al., 1998). For example ephrin-B1 is expressed in the chick retina, and EphB2 isolated from this region is highly phosphorylated on the two juxtamembrane tyrosines (Braisted et al., 1997; Hindges et al., 2002; Kalo and Pasquale, 1999). The juxtamembrane tyrosines have recently been shown to have a major role in regulating the Eph receptor kinase activity (Binns et al., 2000; Wybenga-Groot et al., 2001; Zisch et al., 1998; Zisch et al., 2000) (see section 1.2.3). In addition the juxtamembrane tyrosine residues are docking sites for the SH2 domains of many signalling proteins, including members of the Src family of non receptor tyrosine kinases (Ellis et al., 1996; Hock et al., 1998a; Zisch et al., 1998), Abl (Yu et al., 2001) and RasGAP (Hock et al., 1998a; Holland et al., 1997), as well as adaptor molecules such as Nck and Crk (Hock et al., 1998a; Stein et al., 1998a).*

Centrally located within the Eph receptor cytoplasmic tail is the kinase domain (Ellis et al., 1996; Zisch et al., 1998), which has been shown to interact with the SH2 domains of Grb2 and both subunits of PI3 kinase (Gu and Park, 2001; Pandey et al., 1994; Stein et al., 1996). C-terminal to the catalytic domain is a sterile alpha motif (SAM) domain that may contribute to receptor clustering (Stapleton et al., 1999; Thanos et al., 1999) and a conserved tyrosine motif within the SAM domain is required for interaction with the SH2 domain of Grb10 (Stein et al., 1996) and docking of the low molecular weight protein-tyrosine phosphatase (LMW-PTP) (Stein et al., 1998b). The C-terminal PDZ-binding domain may influence receptor clustering in addition to mediating interactions between Eph receptors and PDZ-containing signalling proteins (Buchert et al., 1999; Cowan et al., 2000; Hock et al.,

Fig 1.1A. Activation of Eph receptor signalling.



A. In the inactive conformation, the Eph receptor juxtamembrane region is involved in a hydrophobic interaction with the kinase domain. **B.** Upon ephrin binding, the two highly conserved juxtamembrane tyrosine residues become phosphorylated, disrupting the intramolecular interaction, allowing the kinase domain to become activated, and inducing phosphorylation at other residues within the Eph receptor intracellular domain. Some of the known Eph receptor binding partners and their interaction positions within the receptor are shown in C, together with the cellular responses these binding partners mediate downstream of active Eph receptors.

1998b; Lin et al., 1999; Torres et al., 1998). The physiological relevance of many of these interactions is unknown, but some have been implicated in Eph receptor signals that converge on the actin cytoskeleton and/or adhesion machinery. For example RasGAP activity has been functionally linked to EphB-mediated neurite retraction, and EphA8-mediated increases in cell adhesion require interactions between EphA8 and the catalytic subunit of PI3 kinase (Elowe et al., 2001; Gu and Park, 2001). Eph receptor signalling cascades are discussed in section 1.5.

1.2.2. Ligand-receptor binding and clustering.

During Eph receptor-ephrin binding the hydrophobic receptor-binding domain of the ephrin molecule tucks into the cleft between two antiparallel β sheets at the N-terminus of the cognate Eph receptor, and the Eph receptor ligand-binding domain then folds to increase the extent of the ligand-receptor interface (Himanen et al., 1998; Himanen et al., 2001). The interaction between side chains at the receptor ligand interface is likely the basis for subclass specificity of ligand binding. Ephrin-Bs have bulky polar residues that are positioned against smaller polar residues in EphB receptors (Himanen et al., 2001). Ephrin-A side chains can be polar or hydrophobic but are small, whereas EphA side chains are large. This means that mixed combinations of receptor-ligand would either result in a hydrophobic residue aligned with a polar residue, or the alignment of two bulky residues, either of which is an energetically unfavourable situation (Himanen et al., 2001).

Isolated extracellular domains of Eph receptors in solution form high affinity heterodimers with ephrins over a large concentration range, but at higher concentrations a tetramer consisting 2 receptor-ligand dimers can form (Himanen and Nikolov, 2002; Lackmann et al., 1997). Crystal structure of an ephrin-B2-EphB2 complex reveals just such a tetrameric structure arranged in a ring with each receptor interacting with 2 ligands, and vice versa (Himanen et al., 2001). Two of the four receptor-ligand interfaces are extensive, and so presumably represent the initial high affinity receptor-ligand interaction. The tetrameric structure is arranged so that the C-terminals of the receptor molecules are on the opposite side of the complex to the C-terminals of the ligand molecules, allowing signals to be transduced in opposite directions at the cell-cell interface (Himanen et al., 2001). The tetrameric arrangement may be relevant to Eph receptor signalling. It has long been known that

ephrin monomers cannot phosphorylate Eph receptors, and unless they are clustered, either artificially or as a consequence of membrane tethering, will not induce a cellular response (Davis et al., 1994). Ephrin-B1 fused to the Fc fragment of human IgG (ephrin-B1-Fc) and presented as a dimer elicits different cellular responses to those induced by ephrin-B1-Fc multimers (Stein et al., 1998b).

1.2.3. Eph receptor activation.

Generally RTKs are activated by ligand binding forcing two catalytically inactive kinase domains into close proximity to favour transphosphorylation. One receptor then phosphorylates regulatory sites on a second receptor, usually within the kinase domain, leading to de-repression of its kinase activity. This in turn allows phosphorylation of other molecules, including the kinase domain of the first receptor, and so a signalling cascade is initiated (van der Geer et al., 1994).

Substitution of the conserved juxtamembrane tyrosine residues with phenylalanine impairs Eph receptor kinase activity against autophosphorylation sites and exogenous substrates, and this observation suggests that these residues have a regulatory function within the Eph receptor (Binns et al., 2000; Wybenga-Groot et al., 2001; Zisch et al., 1998; Zisch et al., 2000). This idea is supported by the fact that mutation of the regulatory tyrosine residue within the kinase domain of EphA4 does not fully inhibit kinase activity, while mutation of the juxtamembrane tyrosines does (Binns et al., 2000). Examination of the crystal structure of EphB2 reveals a hydrophobic interaction between the unphosphorylated juxtamembrane region and the kinase domain, forcing the Eph receptor into an inactive conformation (Wybenga-Groot et al., 2001). Phosphorylation of the juxtamembrane region is thought to disrupt the hydrophobic interaction with the kinase domain and relieve the structural constraints on the activation loop and active site, and consistent with this mutations in which the interaction is artificially disrupted lead to an increase in kinase activity (Wybenga-Groot et al., 2001). In the current model of Eph receptor activation ligand binding brings Eph receptors into sufficiently close proximity to transphosphorylate each other in the juxtamembrane region, which in turn allows the kinase domain to become active and phosphorylate downstream targets. In addition the exposed phosphorylated juxtamembrane tyrosines are made available for binding to SH2-containing molecules.

1.3. Eph receptors and ephrins in axon guidance.

The production of neuronal connections with appropriate targets during embryogenesis is crucial for a functioning adult nervous system, and navigation of a growing neuronal axon to its target during development is dependent on the neuronal growth cone. The growth cone is a highly dynamic structure constructed of a veil-like lamella strung between finger-like filopodia. As the growth cone advances, receptors on its surface respond to guidance molecules in the embryonic environment by initiating signalling cascades that ultimately converge on the cytoskeleton and cellular adhesions (Dickson, 2002; Mueller, 1999).

Axonal guidance cues are categorised as attractive or repulsive depending on the axonal response. Cues that lead to net addition of cytoskeletal components are thought to promote growth cone advance, and are hence classed as attractive guidance cues. Conversely net disassembly of the cytoskeleton by repulsive axon guidance cues may lead to axon retraction (Mueller, 1999; Tessier-Lavigne and Goodman, 1996). Asymmetric signalling across the growth cone can result in the axon turning towards an attractive guidance cue, or away from a repulsive guidance cue. Axonal guidance molecules may be presented on the surface of other cells, or the extracellular matrix, or may be soluble secreted molecules (Mueller, 1999; Tessier-Lavigne and Goodman, 1996).

Ephrins act as contact-dependent axon guidance cues for many types of neuron, and mostly, but not exclusively, induce repulsive guidance signals downstream of axonal Eph receptors. Interestingly reverse signalling through ephrins may also guide axons in some situations.

1.3.1. Topographic mapping of the Retinotectal projection.

During embryonic development many sensory neuronal projections terminate in a spatial order that reflects the arrangement of the neurons from which they arise. This allows the spatial content of information to be preserved between the projecting and target areas. The pre-eminent model for studies on these topographic maps is the projection of retinal axons to the midbrain, where they terminate in the optic tectum (OT) in avians, amphibians and fish, or the superior colliculus (SC) in mammals. Retinal ganglion cell (RGC) axons innervate the OT/SC in a pattern determined by their positions in the retina and this topographic mapping of the

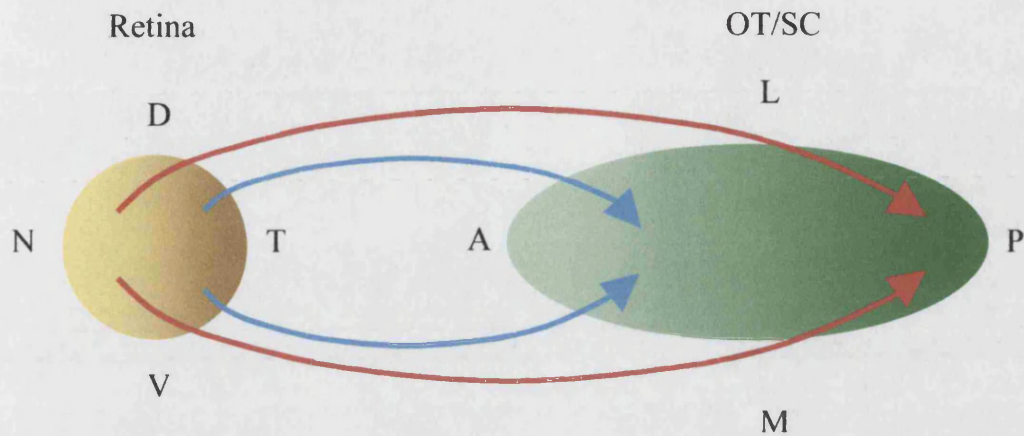
retinotectal/retinocollicular projections occurs along two axes. Axons arising from the temporal retina map to termination zones in the anterior OT/SC, while nasal RGC axons terminate in increasingly posterior positions (Holt and Harris, 1993; Mey and Thanos, 1992) (Fig 1.2). Along the second axis, RGCs from dorsal retina terminate in the lateral OT/SC and ventral RGC axons map to the medial OT/SC (Holt and Harris, 1993; Mey and Thanos, 1992) (Fig 1.2). In chicks, mice and rats, RGCs enter the OT/SC at the anterior pole and grow along the anterior-posterior axis. The primary axons initially overshoot their ultimate target position, and interstitial branches are sent out at the anterior-posterior level corresponding to the final termination position (Hindges et al., 2002; McLaughlin et al., 2003; Nakamura and O'Leary, 1989; Simon and O'Leary, 1992a; Simon and O'Leary, 1992c). These branches then extend laterally or medially towards the termination zone appropriate to the nasal-temporal and dorso-ventral retinal origin, where they finally arborize. Eph receptor-ephrin interactions have been shown to be crucial for all of these steps during development; initial guidance of the primary axon to a particular anterior-posterior position, restriction of the subsequent interstitial branches to appropriate anterior-posterior region, and guidance of the interstitial branches along the lateral-medial axis towards the correct termination zone.

1.3.1.1. Retinotopic mapping along the anterior-posterior axis.

Early studies suggested that the differential distribution of nasal and temporal RGC termination zones in the tectum is a result of differential repulsion of these RGC axons by molecules expressed on the surface of tectal cells (Walter et al., 1987a; Walter et al., 1990b). *In vitro*, temporal axons avoid growing on tectal cell membranes that show no repellent activity for nasal axons (Bonhoeffer and Huf, 1982; Walter et al., 1987a; Walter et al., 1987b; Walter et al., 1990b), and temporal axons respond to soluble tectal membrane portions by collapsing their growth cone and retracting, while nasal RGC axons do not (Cox et al., 1990).

Sperry's chemoaffinity hypothesis suggested that the retinotectal map could result from complementary gradients of receptors and ligands along the nasotemporal retinal and anterioposterior tectal axes, conferring precise positional information for the RGC axons growing into the tectum (Sperry, 1963). EphA receptors and their ephrin-A ligands meet these criteria (Flanagan and Vanderhaeghen, 1998; O'Leary

Figure 1.2. Topographic mapping of the retinotectal projection during development occurs along 2 axes.



RGC axons that originate in the nasal (N) retina terminate in the posterior (P) optic tectum/superior colliculus (OT/SC). Temporal (T) RGC axons terminate in the anterior (A) tectum. Dorsal (D) and ventral (V) RGC axons project to the lateral (L) and medial (M) OT/SC respectively.

and Wilkinson, 1999). EphA3 is expressed in an increasing nasal to temporal gradient in the chick RGC layer (Cheng et al., 1995; Connor et al., 1998; Monschau et al., 1997), while ephrin-A2 and ephrin-A5 are both expressed in an increasing anterior to posterior gradient in the chick tectum (Cheng et al., 1995; Connor et al., 1998; Drescher et al., 1995; Monschau et al., 1997) (Fig 1.3A). Ephrin-A2 is expressed in a shallow gradient across the entire tectum whereas ephrin-A5 is restricted to the posterior half where its expression gradient is relatively steep (Cheng et al., 1995; Drescher et al., 1995; Monschau et al., 1997) (Fig 1.3A).

1.3.1.2. Differential repulsion of RGC projections.

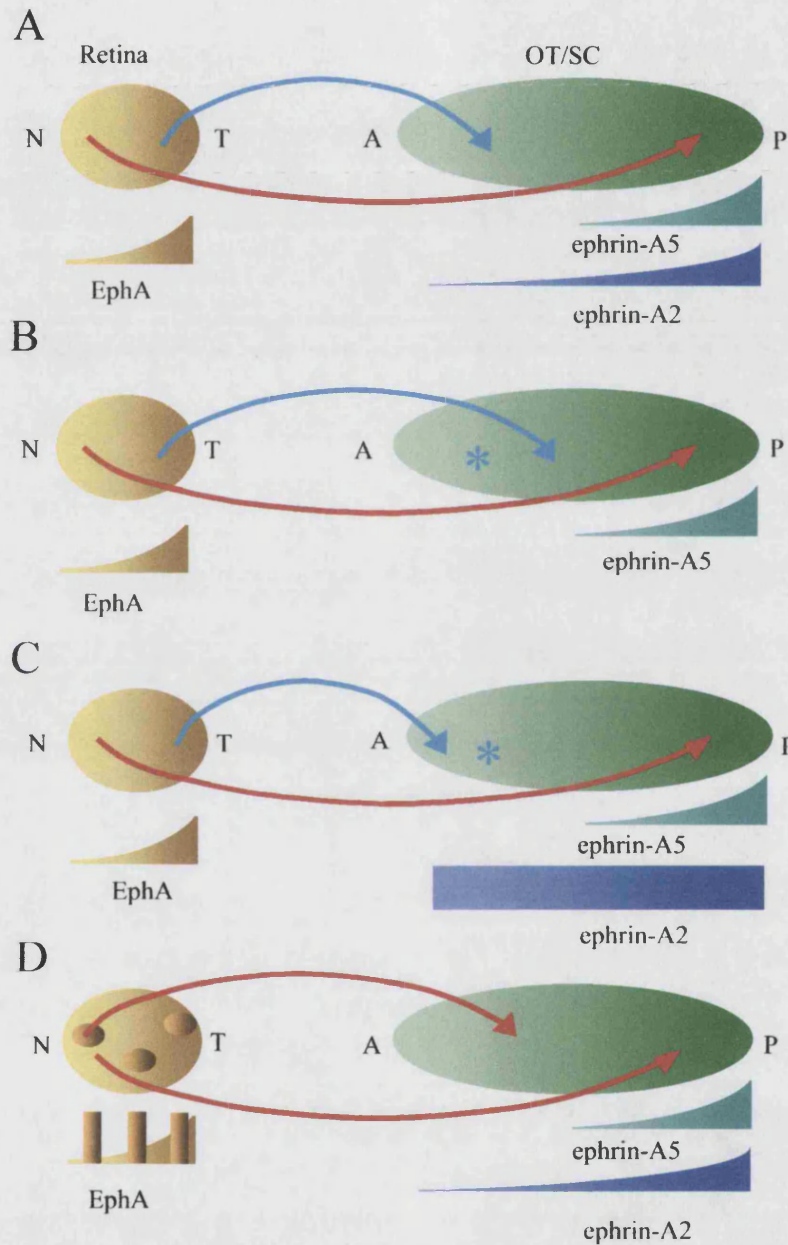
The mapping of temporal RGCs, expressing high levels of EphA receptor, to the anterior region of the tectum which expresses low levels of ephrin-A ligand suggests that repulsive signalling by EphA receptors underlies the retinotopic mapping along the anteroposterior tectal axis. Genetic manipulations of ephrin-A2 and ephrin-A5 expression in the chick and mouse have demonstrated that these molecules govern retinotectal/retinocollicular mapping along the anteroposterior axis by differential repulsion of nasal and temporal RGC projections. Temporal RGCs in mice lacking ephrin-A2 and/or ephrin-A5 project to ectopic termination zones located posterior to

the appropriate position, whereas ephrin-A2^{-/-} nasal RGCs projections are not effected (Feldheim et al., 2000; Frisen et al., 1998) (Fig 1.2B). Conversely expression of an avian retrovirus encoding ephrin-A2 in the anterior tectum, such that the expression level is comparable to that of wild-type posterior tectum, results in temporal RGCs targeting abnormally anterior positions, while nasal RGCs project normally (Nakamoto et al., 1996) (Fig 1.3C).

Ephrin-A2 and ephrin-A5 also differentially repel RGC axons *in vitro*. Ephrin-A2 selectively inhibits outgrowth of temporal axons *in vitro*, and causes temporal growth cone collapse with no effect on nasal axons (Monschau et al., 1997; Nakamoto et al., 1996). Ephrin-A5 is repulsive to both nasal and temporal axons *in vitro*, but again temporal axons are more sensitive to ephrin-A5 than nasal (Nakamoto et al., 1996).

Underlying the difference in sensitivity between nasal and temporal axons to ephrin-A stimulation is the increasing nasal to temporal expression of EphA3, the preferred receptor for ephrin-A2 and ephrin-A5, in the chick retina (Cheng et al., 1995; Connor et al., 1998; Monschau et al., 1997). In the mouse RGCs do not express EphA3. Instead EphA5 and EphA6 are expressed in a nasal to temporal gradient. Expression of EphA3 in small ectopic patches of the mouse retina renders nasal RGCs more sensitive to ephrin-As, causing them to terminate in the anterior SC (Brown et al., 2000) (Fig 1.2D), whereas expression of dominant negative EphA3 induces aberrant temporal RGC projections to the posterior SC (Feldheim et al., 2004). EphA4 also plays a role in the differential sensitivity of RGCs to Ephrin-A ligands. Although expressed uniformly across the nasal-temporal retinal axis, EphA4 is normally highly phosphorylated in nasal but not temporal retina (Connor et al., 1998; Hornberger et al., 1999). The phosphorylation gradient of EphA4 corresponds to the high nasal to low temporal expression of ephrin-As in the retina (Connor et al., 1998; Marcus et al., 1996). Over-expression of ephrin-A5 in the temporal retina leads to corresponding high levels of phosphorylated EphA4 in the temporal retina (Hornberger et al., 1999) suggesting that EphA4 phosphorylation seen in wild-type nasal RGC axons is a consequence of co-expression of ephrin-As and EphA4 on these axons (Hornberger et al., 1999). Persistent phosphorylation of retinal EphA4 inversely correlates with RGC axon sensitivity to exogenous ephrin, probably via a desensitisation mechanism (Hornberger et al., 1999). As described above, nasal RGC axons are normally insensitive to exogenous ephrin-A2 (Monschau et al., 1997;

Figure 1.3. EphA receptors and ephrin-As control retinotectal mapping along the anterior-posterior (A-P) axis.



A, Axons from temporal (T) retina express relatively high levels of EphA receptor and project to regions of the optic tectum/superior colliculus (OT/SC) that express correspondingly low levels of ephrin-A molecules. Nasal (N) RGC axons, which express lower levels of EphA, terminate in the posterior (P) OT/SC. **B**, Temporal RGC axons project to abnormally posterior positions in *ephrin-A2*^{-/-} mice (* indicates appropriate termination zone). **C**, Over-expression of ephrin-A2 in the retina causes temporal RGCs to project to abnormally anterior positions (* indicates appropriate termination zone). **D**, Axons arising from ectopically high areas of EphA receptor expression project to ectopic positions in the anterior tectum.

Nakamoto et al., 1996), but removal of ephrin-A on nasal RGC axons results in their being repelled by substrate-bound ephrin-A2 (Hornberger et al., 1999). Conversely over-expression of ephrin-A5 in the temporal retina abolishes the preferential growth of temporal RGC axons on anterior rather than posterior tectal membranes (Hornberger et al., 1999). Genetic studies have also demonstrated that nasal RGCs from ephrin-A2^{-/-}/A5^{-/-} knockout mice terminate in abnormally anterior positions, and that these axons are more sensitive than wild-type to ephrin-A *in vitro* (Feldheim et al., 2000). Overall the data support a model in which phosphorylation of EphA4 on nasal RGC axons reflects the expression of ephrin-As on neighbouring RGC axons, and by reducing the number of EphA receptors available, this accounts for the lower sensitivity of nasal RGCs to ephrin-As expressed in the tectum, or exogenously added *in vitro*.

These mechanisms play complimentary roles in the differential sensitivity of nasal and temporal RGCs to ephrin-A. Loss of both ephrin-A2 and ephrin-A5 from the retina does not abolish the relative difference in sensitivity of temporal axons to wild type posterior SC membrane, presumably since EphA3 is differentially expressed in these axons (Feldheim et al., 2000). Blocking EphA4 activity abolishes sensitivity of nasal axons to exogenous ephrin-A5, but not temporal axons (Walkenhorst et al., 2000). The apparent gradient of “silenced” EphA4, which results from the ephrin-A gradient in the retina, complements the gradient of expression of EphA3 in the retina.

The above observations have lead to the hypothesis that graded repulsion by tectal ephrin-As sorts the more sensitive temporal axons, which express higher levels of “available” EphA receptors, to areas of low ephrin expression, and less sensitive nasal RGC axons to the posterior tectum. However graded repulsion alone cannot account for the observed phenotype in loss of function ephrin mutants. Knocking out ephrin-A2 and/or ephrin-A5 in the SC does result in the termination zones of temporal RGC axons being shifted in the posterior direction, which agrees with the idea that increasing ephrin-A levels in the posterior colliculus normally act as a repulsive signal (Feldheim et al., 2000; Frisen et al., 1998). However, in the absence of both ephrin-A2 and -A5 many axons project to the appropriate position and RGC axons terminate over the entire SC surface, rather than all being at the posterior pole in the absence of any repellent molecules (Feldheim et al., 2000). A model of graded repulsion together with axon competition has been suggested to explain these results.

Normally temporal axons are repelled from the posterior tectum because of their high sensitivity for ephrin-A, and therefore terminate in the anterior tectum. Nasal axons can navigate into the posterior tectum where competition for an attractive or permissive factor is less. In the absence of ephrin-A the competition between axons results in the retinotopic map filling the entire SC (Feldheim et al., 2000). A computer model that incorporates competition between axons for target sites correctly predicts the topographic maps generated experimentally by genetic manipulation of both Eph receptors and ephrins in the retinotectal system (Honda, 2003).

1.3.1.2.1. Guidance of the primary RGC axon.

As described above the termination zone of a RGC axon is formed by the preferential extension of interstitial branches at the correct anterioposterior position once the primary axon has overshoot, and recently evidence has begun to accumulate suggesting that EphA receptor-ephrin-A interaction restricts axonal branching to the correct A-P level (Yates et al., 2001) (see section 1.3.1.2.2). Early studies investigating the role of EphA-ephrin-A interaction during retinotopic mapping concentrated on the position of RGC termination zones in the tectum, but did not distinguish between the role of EphA-ephrin-A interaction in guidance of the primary axon from subsequent effects on axon branching. The *in vitro* experiments described above together with recent *in vivo* evidence demonstrate that EphA-ephrin-A interaction effects the guidance of the primary axon by differential repulsion.

Although all axons overshoot their ultimate target position along the anterior-posterior axis, there is some retinotopic specificity in the initial targeting of RGC axons, since temporal RGC axons initially terminate in more anterior positions in the tectum than nasal RGC axons (Nakamura and O'Leary, 1989; Yates et al., 2001). It seems likely that the primary RGC axons are guided within the tectum by EphA-mediated repulsion since temporal RGC axons *in vivo* change their trajectory to avoid ectopic patches of abnormally high ephrin-A2 expression in the anterior tectum (Nakamoto et al., 1996). In addition temporal axons that over-express ephrin-A2 or ephrin-A5, and are therefore less sensitive to ephrin-A stimulation (see above), initially extend further along the A-P axis than wild type temporal RGC axons (Dutting et al., 1999; Hornberger et al., 1999).

More recently the technique of chromophore-assisted laser inactivation (CALI) has revealed a role for tectal ephrin-A5 in the repulsion of RGC primary axons (Sakurai et al., 2002). The principal of CALI is that irradiation of a chromophore-labelled antibody leads to specific local inactivation of the targeted protein to which the antibody is bound (Beermann and Jay, 1994). Inactivation of ephrin-A5 in specific areas within the posterior tectum leads to nasal RGC axons that would normally terminate in the irradiated region overextending into more posterior areas (Sakurai et al., 2002). Taken together the above is evidence for a role of EphA-ephrin-A interaction in the guidance of RGC axons *in vivo* by a mechanism of graded repulsion.

1.3.1.2.2. Restriction of interstitial branches.

The bias for RGC axons to initiate and extend branches at the appropriate anteroposterior level within the tectum is mirrored *in vitro*, as temporal RGC axons extend branches on stripes of anterior rather than posterior tectal membrane (Yates et al., 2001). This preference is abolished by addition of soluble EphA3, suggesting that tectal ephrin-As normally act to inhibit RGC axon branching (Yates et al., 2001). Inactivation of ephrin-A5 in specific regions of the tectum by CALI has revealed a major role of EphA-ephrin-A interaction in restricting RGC axons to the correct anteroposterior position *in vivo*. Removal of ephrin-A5 in the posterior tectum results in axons that would normally project to the irradiated area branching at positions posterior to the correct termination zone suggesting that branch elongation is normally restricted by tectal ephrin-A5 (Sakurai et al., 2002).

If RGC axons are prevented from branching in too posterior a position by high levels of ephrin-A expression, an additional signal must exist that prevents axon branching in the anterior tectum where ephrin-A expression is low (Yates et al., 2001). It has been suggested that one possibility is that ephrin-A reverse signalling is responsible for inhibiting axonal branching in the anterior tectum (Yates et al., 2001), since nasal RGCs express high levels of ephrin-A2 and ephrin-A5 (Connor et al., 1998; Hornberger et al., 1999; Marcus et al., 1996) and EphA3 is present at high levels in the anterior tectum (Cheng et al., 1995; Connor et al., 1998; Monschau et al., 1997). However, it has recently been shown that EphA3-induced reverse signalling by both ephrin-A2 and ephrin-A5 leads to increased adhesion (Huai and Drescher, 2001). Interstitial branches initially appear as filopodial-like structures, and since adhesion

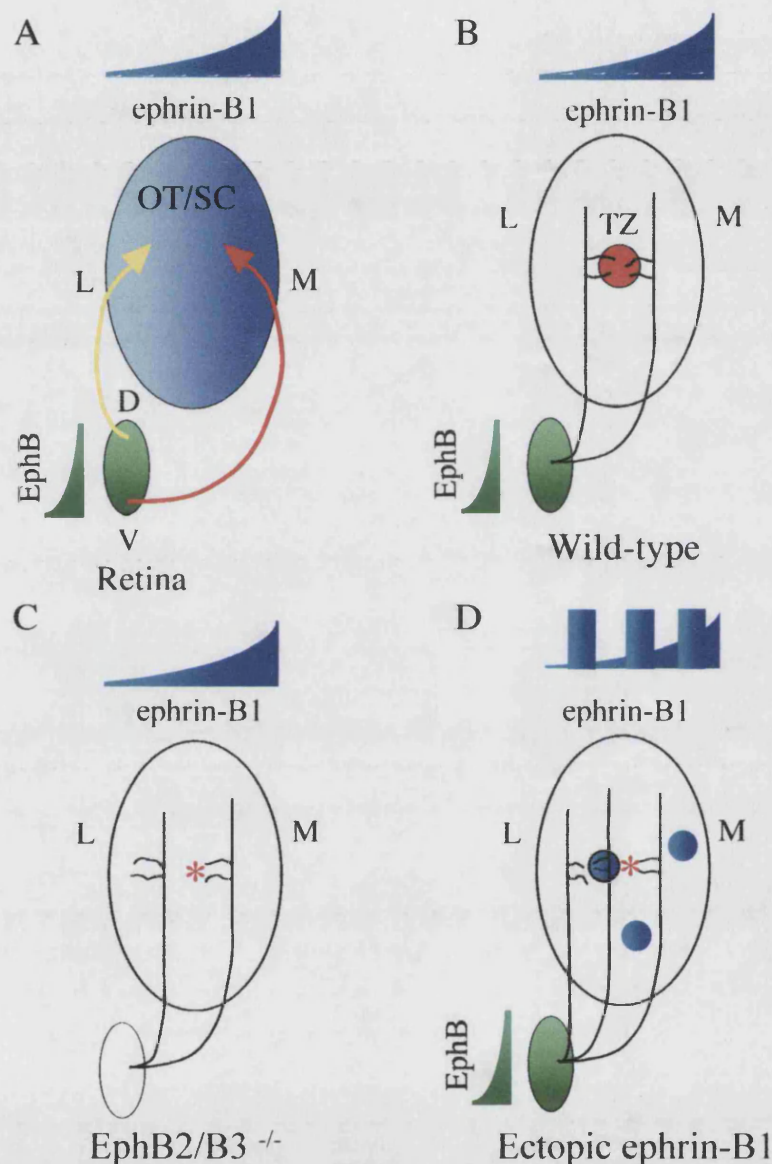
to the substrate is necessary for filopodial stability (Varnum-Finney and Reichardt, 1994), it would be predicted that high levels of ephrin-A reverse signalling in the anterior tectum would act to promote the stabilisation of newly formed branches by increasing adhesion. It is therefore unlikely that ephrin-A reverse signalling restricts RGC axons from branching in inappropriately anterior positions.

1.3.1.3. Retinotopic mapping along the lateral-medial axis.

Ventral RGCs expressing high levels of EphB2 project to the medial tectum, where there is a high expression of ephrin-B1, and dorsal RGCs, expressing lower levels of EphB2 terminate in the lateral tectum (Fig 1.4A) suggesting an attractive interaction between EphB2 and ephrin-B1 (Braisted et al., 1997).

In the mammalian and avian systems RGCs from a specific dorso-ventral position in the retina enter the optic tectum or superior colliculus over a broad lateral-medial range and extend interstitial branches towards their appropriate termination zone (Hindges et al., 2002; Simon and O'Leary, 1992b). RGCs that are medial to their termination zones preferentially extend interstitial branches laterally, down the ephrin-B1 gradient, whereas RGCs that are lateral to their termination zone preferentially extend interstitial branches medially, up the ephrin-B1 gradient (Fig 1.4B). In EphB2/B3 knockout mice branches always grow laterally down the ephrin-B1 gradient, regardless of whether the axon is lateral or medial to its termination zone (Fig 1.4C), suggesting that they have lost the ability to respond to an attractive signal from the higher levels of ephrin-B1 in the medial SC (Hindges et al., 2002). The ephrin-B1-induced attractive signal is mediated by EphB forward signalling, since dominant negative EphB2 expression results in similar defects (Hindges et al., 2002). However, if ephrin-B1-induced attraction is the only signal governing the direction of interstitial branch extension in the wild-type animal, EphB2-EphB3 knockouts should show interstitial branches extending randomly (Hindges et al., 2002; McLaughlin et al., 2003). Instead the EphB2/EphB3 phenotype, in which all branches grow laterally, suggests that a repulsive signal acts to guide branches laterally in the knockout mice (Hindges et al., 2002; McLaughlin et al., 2003). Ectopic expression of ephrin-B1 in patches, superimposed on the wild-type ephrin-B1 gradient in the chick tectum, results in regions of ectopically high but still graded ephrin-B1 expression (Fig 1.4D) (McLaughlin et al., 2003). Within

Fig 1.4. EphB-ephrin-B interaction controls retinotectal mapping along the lateral-medial axis.



A. There is an increasing D to V gradient of EphB receptors in the retina and a corresponding increasing L to M gradient of ephrin-B1 in the OT/SC. RGCs arising from the dorsal (D) retina project to the lateral (L) optic tectum/superior colliculus (OT/SC), whereas ventral RGC axons terminate in the medial (M) OT/SC. **B.** In the wild-type visual system RGC axons arising from the same position in the retina enter the OT/SC over a broad L-M range. RGC axons medial to their correct target zone (TZ) grow laterally, down the ephrin-B1 gradient, and RGC axons lateral to their TZ grow medially, up the ephrin-B1 gradient. **C.** In EphB2^{-/-}B3^{-/-} knockout mice all RGC axons extend branches laterally, regardless of their L-M position (* indicates appropriate TZ). **D.** RGC axons lateral to their TZ extend branches laterally in areas of ectopically high ephrin-B1 expression (* indicates appropriate TZ).

these patches all interstitial branches extend down the ephrin-B1 gradient, regardless of whether they are lateral or medial to their termination zone (Fig 1.4D) (McLaughlin et al., 2003). The above data support a model in which high levels of ephrin-B1 are repulsive to interstitial branches, and therefore axons medial to their termination zone (in a position of inappropriately high ephrin-B1 expression) preferentially extend branches down the ephrin-B1 gradient. In this model lower levels of ephrin-B1 are attractive and therefore branches from axons lateral to their correct termination zone preferentially extend branches medially, up the ephrin-B1 gradient (McLaughlin et al., 2003).

It is possible that different EphB receptors mediate the attractive and repulsive responses to ephrin-B1 in the OT/SC (McLaughlin et al., 2003). EphB2/B3 null RGC axons are still directed down the ephrin-B1 gradient, suggesting they still respond to ephrin-B1 as a repulsive cue (Hindges et al., 2002). EphB1, which is uniformly expressed across the retina, has been shown to recruit different signalling complexes to EphB2 when stimulated with ephrin-B1, and this may underlie a differential response of wild-type and EphB2/EphB3 null RGC axons to the ephrin-B1 gradient in the superior colliculus (McLaughlin et al., 2003; Stein et al., 1998b). Clustering of ephrin-B1 into dimers has been shown to cause different responses and recruitment of alternate signalling complexes compared with ephrin-B1 tetramers (Huynh-Do et al., 1999; Stein et al., 1998b). Increasingly high order clusters of ephrin-B1 *in vitro* may mimic the increasing ephrin-B1 gradient in the OT/SC, and therefore contribute to the switch in response of RGCs from attraction to repulsion (McLaughlin et al., 2003).

1.3.2. Topographic mapping of the Hippocamposeptal projection.

Countergradients of EphA and ephrin-A expression are thought to underlie topographic specificity of neuronal connections other than the retinotectal projection. For example at the time when hippocampal axons are reaching their target positions in the septum, a low medial to high lateral gradient of ephrin-A5 expression is seen within the septum (Zhang et al., 1996a). Axons arising from the medial hippocampus, which expresses multiple EphA receptors (Gao et al., 1996; Yue et al., 2002; Zhang et al., 1996a), project to the medial septum whereas the lateral hippocampus, which displays weak EphA expression, innervates the lateral septum.

In vitro ephrin-A5 has selective inhibitory effects on the outgrowth of medial, but not lateral hippocampal axons (Brownlee et al., 2000) and expression of a dominant negative EphA5 allows projections from medial hippocampal axons to invade the more lateral septum, from which they are normally excluded (Yue et al., 2002).

Interestingly the pattern of hippocampal projections observed in animals expressing dominant negative EphA5 suggests that inter-axon competition may play a role in topographic targeting in the Hippocamboseptal system (Yue et al., 2002), as is thought to occur in the retinotectal system. It is also intriguing that, while inhibitory effects on neurites *in vitro* are seen after a fixed time (Brownlee et al., 2000), careful analysis of axons demonstrates that short (<24 hour) exposure to ephrin-A5 increases hippocampal axon length, and axon retraction and fragmentation occur only after prolonged exposure (Gao et al., 1999), suggesting that ephrin-A5 might have bifunctional effects on these axons.

1.3.3. Topographic mapping of the Vomeronasal Projection.

In terrestrial vertebrates the vomeronasal organ is primarily responsible for detecting pheromones. Vomeronasal axons travel along the septum and across the surface of the main olfactory bulb to terminate in their target, the accessory olfactory bulb. The axons reaching the accessory olfactory bulb are segregated according to their origin in the vomeronasal organ, and basal vomeronasal axons innervate the posterior accessory olfactory bulb, while apical axons project to the anterior accessory olfactory bulb.

EphA-ephrin-A interaction has again been implicated in the formation of this topographic map, but in this case vomeronasal axons appear to be guided by attractive, reverse signals mediated by ephrin-As. Ephrin-A5 is more highly expressed in apical than basal vomeronasal neurons, and EphA6 is present at high levels in the anterior accessory olfactory bulb (Knoll and Drescher, 2002). In mice lacking ephrin-A5, apical vomeronasal axons project to both the anterior and posterior accessory olfactory bulb, suggesting that they have lost the ability to respond to a graded signal. Since vomeronasal axons preferentially grow on EphA substrates *in vitro*, it is likely that the accessory olfactory bulb signal is normally attractive (Knoll and Drescher, 2002).

1.3.4. Non-graded ephrin-Eph expression.

In many regions of the nervous system Eph receptor-ephrin interaction appears to influence axon trajectories by limiting growth to particular Eph receptor/ephrin territories, or along Eph-ephrin boundaries. For example, ephrin expression at the midline appears to be very important for the correct guidance of both ascending and descending axon tracts, and within the brain, regional ephrin expression restricts the path taken by certain axons.

1.3.4.1. Guidance of Corticospinal tract axons.

Mice that are null for EphA4 or ephrin-B3 are grossly normal, but suffer from a very specific neurological defect in that they are incapable of unilateral movements. Most noticeably EphA4 and ephrin-B3 knockout mice walk with a hopping gait as both forepaws move together, as do both hind paws (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). Retrograde and anterograde labelling of CNS neurons in these mice reveal that they have a defective projection of the corticospinal tract, which is the major descending pathway that controls voluntary movement. In normal animals all the corticospinal axons cross the midline in the medulla and project to the contralateral spinal grey matter. In the mutant mice corticospinal axons are able to re-cross the midline leading to bilateral innervation of the spinal cord (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001).

Ephrin-B3 is expressed along the midline of the developing embryo (Gale et al., 1996a; Imondi et al., 2000; Kullander et al., 2001a; Kullander et al., 2001b; Yokoyama et al., 2001) where it appears to act as a repulsive barrier for the EphA4 expressing corticospinal axons (Kullander et al., 2001b; Yokoyama et al., 2001). *In vitro* neurons from the cortical region in which the corticospinal tract originates collapse in response to ephrin-B3 and within the medulla ephrin-B3 expression is limited to the dorsal region, whereas the tracts cross the midline ventrally (Kullander et al., 2001a). Since mice expressing a truncated EphA4 still exhibit the hopping gait, forward signalling through EphA receptors seems to be required for axons to respect the ephrin-B3 midline barrier and not re-cross the midline (Kullander et al., 2001b).

1.3.4.2. Guidance of Commissural Spinal Interneurons.

In vertebrates, interneurons in the dorsal spinal cord initially send axons ventrally to the floor plate, before crossing the midline and projecting longitudinally. In part, guidance of these commissural axons ventrally is mediated by Netrin secreted from the ventral floorplate, which acts as an attractive cue to the commissural axons (Fazeli et al., 1997; Serafini et al., 1996), but this raises the question of how the axons overcome the attractive floorplate signal in order to continue their trajectory in the contralateral spinal cord.

Ephrin-B1 and ephrin-B3 are expressed in the floor plate of the mouse and the chick throughout the time of commissural axon pathfinding (Flenniken et al., 1996; Gale et al., 1996a; Imondi et al., 2000; Imondi and Kaprielian, 2001) and these ligands cause growth cone collapse of mice and chick commissural axons *in vitro* (Imondi et al., 2000; Imondi and Kaprielian, 2001). Interestingly it has been shown that in both mice and chick EphB receptors are only expressed on sections of commissural axons that have crossed the midline (Imondi et al., 2000; Imondi and Kaprielian, 2001), which presumably allows the axons to enter the ipsilateral floor plate before being restricted to the contralateral spinal cord.

This observation reveals parallels with axon guidance at the midline in *Drosophila*. Roundabout (Robo), which is the receptor for the midline axon repellent Slit, is only expressed on commissural axons once they have crossed the midline (Kidd et al., 1998a). Loss of the Robo gene results in commissural axons re-crossing the midline many times (Kidd et al., 1998a). Although disruption of ephrin-B-EphB interaction does not lead to re-crossing of commissural axons in the mouse (Imondi and Kaprielian, 2001), it has recently been shown that mammalian commissural axons also become sensitive to slit after they have crossed the midline (Zou et al., 2000), suggesting these repulsive guidance cues may act together to constrain commissural projections. Genetic evidence shows that the dynamic regulation of Robo expression is under the control of Commissureless, which is also expressed at the midline (Kidd et al., 1998b). How EphB expression is regulated in commissural axons remains to be investigated.

Careful analysis of the trajectory taken by commissural axons once they have crossed the midline reveals that the majority of axons turn rostrally and grow longitudinally while being gradually deflected dorsally. These axons then make a final rostral turn

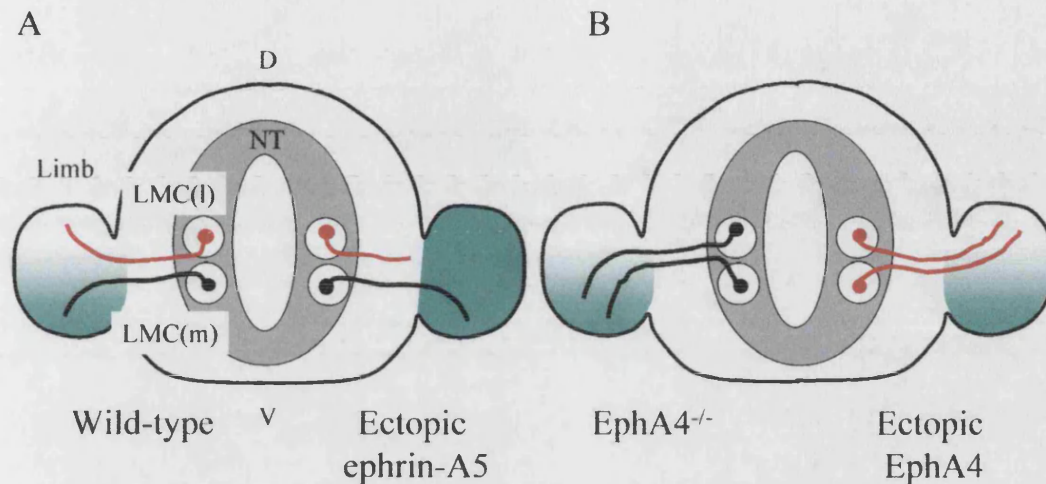
at the border of a region of ephrin-B expression dorsal to their path (Imondi and Kaprielian, 2001). Disruption of ephrin-B-EphB interaction with soluble EphB-Fc prevents commissural axons from making their final rostral turn, and instead they grow into the ephrin-B expressing region (Imondi and Kaprielian, 2001).

1.3.4.3. Guidance of spinal motor axons.

Motor axons arising from the medial lateral motor column [LMC(m)], which do not express EphA4 innervate the ephrin-A5-positive ventral limb, whereas EphA4-expressing LMC [LMC(l)] axons project to the dorsal hind limb, which does not express ephrin-A5 (Eberhart et al., 2000; Eberhart et al., 2004; Ohta et al., 1996) (Fig 1.5A). Ectopic expression of ephrin-A5 in the dorsal hind limb results in LMC(l) axons stalling at the base of the limb (Fig 1.5A), but in EphA4 knock-out mice these axons project with the LMC(m) axons into the ephrin-A5-positive ventral limb (Fig 1.5B), suggesting that EphA4 expression on LMC(l) axons mediates a repulsive response to ephrin-A5 *in vivo* (Eberhart et al., 2004; Helmbacher et al., 2000). In support of this hypothesis ectopic EphA4 expression in LMC(m) axons results in their avoiding the ephrin-A5-positive ventral limb, and instead the axons innervate the dorsal limb (Fig 1.5B) (Eberhart et al., 2000).

In contrast to the behaviour of LMC(l) axons, EphA4-expressing motor axons originating in the medial portion of the medial motor column (MMC(m)) specifically project through the anterior half somite, which is ephrin-A5 -positive. Expression of dominant-negative EphA4 in these axons, or ectopic expression of ephrin-A5 in the posterior half somite, causes aberrant projection into the posterior somite (Eberhart et al., 2004), which suggests that ephrin-A5 is normally attractive to MMC(m) axons. The different responses of EphA4-expressing LMC(l) and MMC(m) axons to ephrin-A5 are conserved *in vitro*. LMC(l) neurons extend very few neurites on an ephrin-A5 substrate, whereas MMC(m) axons exhibit extensive neurite growth on ephrin-A5 compared to control (Eberhart et al., 2004). These results, suggesting that ephrin-A5 may act as a bifunctional guidance cue for motor axons, complement previous work that suggests hippocampal axons show both attractive and repulsive responses to ephrin-A5 (Gao et al., 1999; Brownlee et al., 2000; see section 1.3.2), and that ephrin-B1 has both attractive and repulsive guidance effects on RGC axons

Figure 1.5. EphA receptors and ephrin-As control the guidance of spinal motor neurons.



A. In wild-type animals EphA4-expressing axons (red) arising in the lateral part of the lateral motor column [LMC(l)] project to the dorsal (D) limb, which does not express ephrin-A5, but EphA4-negative axons (black) from the medial LMC [LMC(m)] innervate ephrin-A5-positive (green) territory in the ventral (V) limb. Ectopic ephrin-A5 expression in the D limb causes LMC(l) axons to stall at the base of the limb. **B.** LMC(l) axons in an EphA4 knockout animal project with the LMC(m) axons into ephrin-A5-positive territory in the V limb, and ectopic expression of EphA4 in the LMC(m) causes these axons to avoid the ephrin-A5-positive territory, and project along with the LMC(l) axons into the D limb. NT = neural tube

(McLaughlin et al., 2003; see section 1.3.1.2) and neural crest cells (Santiago and Erickson, 2002; see section 1.4.2.2).

1.4. Eph Receptors and ephrins in morphogenesis.

Correct development of a multicellular organism requires co-ordinated cell movements, which in turn require synchronised changes in cellular cytoskeleton and adhesion machinery. Eph receptor-ephrin interaction is essential for many of the tissue patterning and morphogenesis events that give rise to a structured embryo.

1.4.1. Development of the vascular system.

Formation of the vascular system during embryogenesis comprises two distinct processes, vasculogenesis and angiogenesis. During vasculogenesis the dispersed populations of vascular endothelial cell (EC) precursors, which will eventually line

the blood vessels, differentiate and proliferate to form an initial network called the primary capillary plexus (Risau and Flamme, 1995). During angiogenesis the existing vessels of the primary capillary plexus are remodelled into a mature network via sprouting of new vessels and splitting, branching and pruning of existing vessels (Risau, 1997). Since co-ordinated changes in migration and adhesion of ECs are required for angiogenic remodelling it is unsurprising that Eph receptor-ephrin signalling has been shown to be critical for angiogenesis.

Ephrin-A1 expression is induced by vascular endothelial growth factor (VEGF) stimulation and, like VEGF, has been shown to have angiogenic properties. Ephrin-A1 stimulation induces human umbilical vein endothelial cells (HUVECS) to form capillary-like structures *in vitro*, and implantation of an ephrin-A1-Fc-coated pellet into the rat cornea induces vessel sprouting (Cheng et al., 2002a; Daniel et al., 1996; Ogawa et al., 2000; Pandey et al., 1995b). Despite the observation that ephrin-As are expressed at sites of vascular development in the mouse (Flenniken et al., 1996; McBride and Ruiz, 1998), no vascular defects have been reported in animals lacking a variety of EphA receptors or ephrin-As (Chen et al., 1996; Dottori et al., 1998; Feldheim et al., 2000; Frisen et al., 1998). EphB-ephrin-B signalling has also been implicated in angiogenic processes *in vitro*. Ephrin-B1 can induce capillary-like assembly of ECs (Stein et al., 1998b), and ephrin-B2 can stimulate vessel sprouting in a corneal assay (Maekawa et al., 2003). Unlike ephrin-As however, there is a large body of evidence demonstrating that ephrin-B-EphB receptor signalling is essential for normal angiogenesis.

1.4.1.1. EphB4-ephrin-B2 expression during vascular development.

EphB4 and its ligand, ephrin-B2, show a striking reciprocal expression pattern in the developing vasculature, in that ephrin-B2 is expressed exclusively on arteries and EphB4 is preferentially expressed on veins (Adams et al., 1999; Adams et al., 2001; Gerety et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998)

Inactivation of either ephrin-B2 or EphB4 in mice leads to the identical phenotype of embryonic lethality following the failure of the primary plexus to remodel and veins and arteries to intercalate (Adams et al., 1999; Adams et al., 2001; Gerety et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998). The defects are specific to angiogenesis since the major trunk vessels, formed by vasculogenesis, appear normal

in animals lacking ephrin-B2 or EphB4 (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Interestingly in the ephrin-B2 or EphB4 knockout, defects are seen in both arteries and veins (Adams et al., 1999; Gerety et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998), suggesting that bi-directional signalling between the EphB4- and ephrin-B2- expressing cells is necessary for angiogenesis. This idea is supported by the report that knock-in of an ephrin-B2 construct that lacks the intracellular domain does not rescue the vascular defects seen in ephrin-B2 null mice (Adams et al., 1999). *In vitro* sprouting of ECs is stimulated by addition of both ephrin-B2-Fc and EphB4-Fc, demonstrating that both forward and reverse signalling can lead to cellular changes associated with angiogenesis *in vivo* (Adams et al., 1999; Adams et al., 2001).

It should be noted that, in addition to EphB4, EphB3 is also expressed on embryonic veins in the mouse (Adams et al., 1999). However, several lines of evidence suggest that EphB4 is primarily responsible for correct angiogenesis. EphB3 knock-out mice show no vascular defects (Adams et al., 1999) and while a double knock-out of EphB3/EphB2 results in vascular defects, the phenotype is, intriguingly, less severe than in ephrin-B2 or EphB4 knock-outs, with lower penetrance (Adams et al., 1999). Ephrin-B2 is the only known ligand for EphB4, and although ephrin-B1 appears to be co-expressed with ephrin-B2 on arteries, over-expression of ephrin-B1 cannot rescue ephrin-B2 vascular defects (Wang et al., 1998).

1.4.1.2. EphB4-ephrin-B2 signalling at the venous-arterial boundary.

Despite the crucial role of EphB4 and ephrin-B2 during angiogenesis little is known about the consequences of the complimentary expression pattern of this receptor-ligand pair at a cellular level. One possible role of complimentary ephrin-B2-EphB4 expression in the developing vascular system is to keep the venous and arterial cells separate, analogous to the role of Eph receptor-ephrin signalling in restricting cell intermingling in the hindbrain (see section 1.4.3.1). Endothelial cell lines expressing EphB4 or ephrin-B2 will segregate when mixed (Fuller et al., 2003) suggesting that Eph receptor-ephrin interaction results in a repulsive signal preventing intermingling of arterial and venous cells. Forward signalling through EphB4 is sufficient for this segregation to occur (Fuller et al., 2003). Since truncated ephrin-B2, which would still be capable of initiating forward signalling through EphB4, cannot rescue the

vascular defects seen in ephrin-B2 knockout mice (Adams et al., 1999), it seems unlikely that segregation of venous and arterial cell populations is sufficient to allow normal angiogenesis.

The function of EphB4 forward signalling in EC migration and angiogenic sprouting is not clear. Soluble ephrin-B2-Fc addition, or contact with ephrin-B2 expressing cells has been shown to promote migration of endothelial cells and increase capillary sprouting (Adams et al., 2001; Steinle et al., 2002; Zhang et al., 2001). However soluble ephrin-B2-Fc has also been reported to inhibit endothelial cell migration and sprouting (Fuller et al., 2003). Interpretation of these results is further complicated by the failure to address whether the experimental cell types used express Eph receptors other than EphB4. Interestingly two studies using cells that express EphB4 alone both demonstrate that EphB4 forward signalling inhibits adhesion, migration and sprouting of endothelial cells (Fuller et al., 2003; Hamada et al., 2003).

Contradictory reports have also been published regarding the effects of stimulating ephrin-B2 reverse signalling in endothelial cells. Soluble EphB4-Fc has been shown to stimulate sprouting and migration of arterial endothelial cells, but contact with EphB4 expressing cells inhibits the formation of capillary-like structures by embryonic endothelial cells (Adams et al., 1999; Fuller et al., 2003; Zhang et al., 2001).

1.4.1.3 Sites of neovascularization in the adult.

Recently it has been shown that the reciprocal pattern of ephrin-B2 expression in arteries and EphB4 expression in veins persists in the adult (Gale et al., 2001; Shin et al., 2001). In addition ephrin-B2 is expressed on newly sprouted vessels during wound healing in the adult, and during maturation of the corpus luteum in the adult ovary (Gale et al., 2001; Shin et al., 2001).

The role of Eph receptor-ephrin signalling in the formation of new blood vessels may explain the link between Eph receptors and ephrins, and tumourogenesis (Cheng et al., 2002a). Ephrin-B2 is seen on new blood vessels stimulated by tumourogenesis, and blocking Eph receptor activity has been shown to inhibit tumour vasculogenesis (Brantley et al., 2002; Cheng et al., 2002b)

1.4.1.4. Cross-talk with other mediators of vascular development.

In addition to ephrins, VEGFs and angiopoietins are growth factor families critical for angiogenesis. Mesenchymal cells surrounding the embryonic vessels express angiopoietin-1 (Ang-1), and ECs themselves express the receptor for Ang-1, Tie-2. It has been shown that Tie-2 can directly phosphorylate ephrin-B1, which is expressed on endothelial cells in both veins and arteries (Adams et al 1999), and this may regulate ephrin-B1 signalling. Functionally, ephrin-Eph receptor signalling may interact with other angiogenic signals, since soluble EphA2 can inhibit VEGF-stimulated endothelial cell migration *in vitro*, and neovasculogenesis *in vivo* (Cheng et al 2002).

1.4.2. Somitogenesis and trunk neural crest migration.

A basic feature of all vertebrate embryos is segmentation along the longitudinal body axis. The most striking regions of segmentation are the hindbrain and the paraxial mesoderm, which in turn underlie the development of a segmented nervous system. Eph receptor-ephrin interaction has been shown to play a major role in the induction and maintenance of a segmented body pattern in the embryo.

1.4.2.1. Somitogenesis.

Somites are transient embryonic structures that arise from the segmentation of the paraxial mesoderm into paired blocks. Somites organise the segmental pattern of the peripheral nervous system by restricting the migration of neural crest cells (section 1.4.2.2) and motor axons (section 1.3.4.3), and then give rise to the vertebrae and ribs, the skeletal musculature of the back and limbs and the dorsal dermis. At the onset of somitogenesis the presomitic mesoderm (PSM) begins to express genes in a segmental pattern, and the presumptive somitic tissue is specified as having anterior or posterior identity. The juxtaposition of anterior and posterior PSM leads to the formation of a morphologically distinct intersomitic furrow, and the cells undergo a mesenchymal to epithelial transformation (Maroto and Pourquie, 2001). The genetic factors that underlie the early segmental patterning of PSM are well known (Maroto and Pourquie, 2001; Pourquie, 2001), and Eph receptor-ephrin signalling has been shown to play a role in the morphological changes and epithelialisation that occurs during somitogenesis.

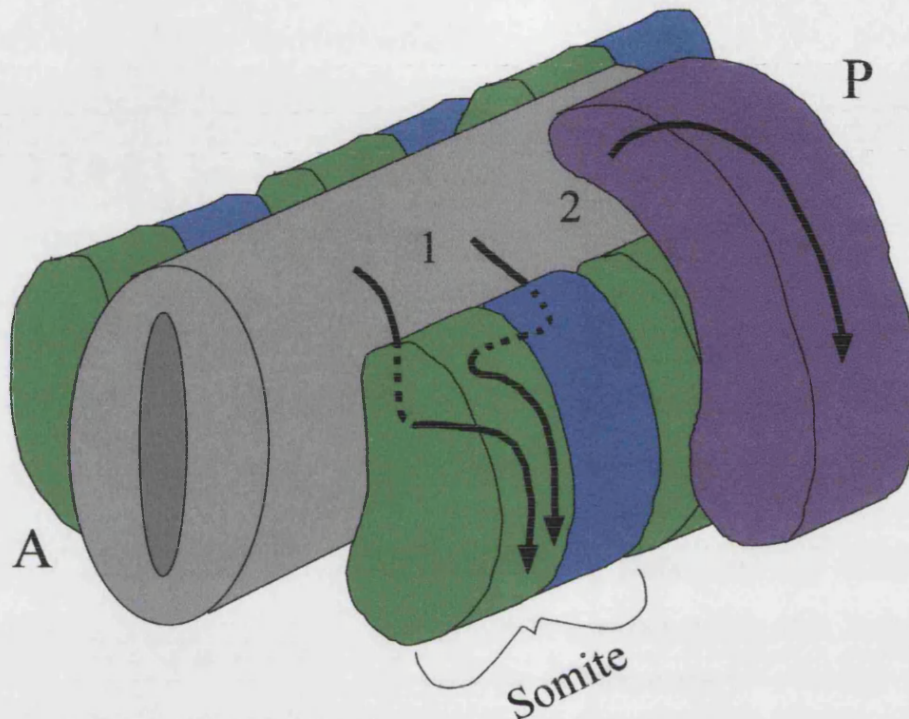
In the zebrafish EphA4 is expressed in the anterior half of the presumptive and newly formed somite, and there is reciprocal expression of two of its ligands in the posterior half of the presumptive somite (Durbin et al., 1998). Disruption of endogenous EphA4-ephrin signalling, by expression of dominant negative EphA4, interferes with somitogenesis by preventing the formation of intersomitic furrows (Durbin et al., 1998). By contrast, transplantation of EphA4-expressing cells into zebrafish mutants that lack somites leads to formation of morphologically distinct intersomitic boundaries between EphA4 expressing- and wild-type cells (Barrios et al., 2003). EphA4-ephrin signalling is necessary for the changes in cell morphology and adhesion that underlie epithelialisation during zebrafish somitogenesis (Barrios et al., 2003), and EphA4 expression in the chick PSM correlates with epithelial-like cell morphology (Schmidt et al., 2001).

1.4.2.2. Trunk neural crest migration.

Trunk neural crest cells delaminate from the neural tube and migrate in one of two spatially and temporally distinct pathways (Fig 1.6). Early migrating neural crest cells (NCCs) migrate ventrally from the neural tube through the anterior half of the somitic sclerotome (pathway 1 in Fig 1.6). These NCCs will become the sensory and sympathetic neurons of the peripheral nervous system, and the restriction of their migration through the anterior somites underlies the segmental arrangement of dorsal root and sympathetic ganglia in the adult animal. The posterior half of the sclerotome and overlying dermomyotome, from which the early NCC are excluded, express ephrin-B ligands (predominantly ephrin-B1 in avians and ephrin-B2 in rodents) (Koblar et al., 2000; Krull et al., 1997; Wang and Anderson, 1997) and the first wave of NCCs express EphB receptors (EphB3 in avians, EphB2 in rodents) (Krull et al., 1997; Wang and Anderson, 1997).

Addition of soluble ephrin-B1-Fc to whole-trunk explants *in vitro*, which will bind to the EphB receptors expressed on NCCs and prevent interaction with endogenous ephrin-B ligands, perturbs the *in situ* migration pattern of early NCCs (Koblar et al., 2000; Krull et al., 1997; Santiago and Erickson, 2002). Disrupting EphB-ephrin-B interaction in this way results in early NCCs migrating through both anterior and posterior halves of the somite, and straying into the overlying dermomyotome (Koblar et al., 2000; Krull et al., 1997; Santiago and Erickson, 2002). Early NCCs *in*

Figure 1.6. Trunk NCCs migrate from the neural tube along two distinct pathways during development.



Pathway 1. Early NCCs delaminate from the neural tube (NT; grey) and migrate ventrally through the anterior (A) half of the adjacent somite (green). NCCs are excluded from entering the posterior (P) half somite (blue) and NCCs initially adjacent to a posterior half somite migrate longitudinally until they are able to navigate through permissive anterior somitic tissue. **Pathway 2.** Later migrating melanoblasts travel dorsolaterally through the overlying dermomyotome (purple).

vitro avoid growing on immobilised ephrin-B1 or ephrin-B2 (Krull et al., 1997; Wang and Anderson, 1997) suggesting that ephrin-B ligands are responsible for a repulsive signal that excludes EphB expressing early NCCs from the posterior sclerotome and dermomyotome.

Approximately 18 hours after the early NCCs begin to migrate from the neural tube, a second wave of NCCs, destined to become melanocytes, migrates dorsolaterally from the neural tube through the dermomyotome (path 2 in Fig 1.7). These melanoblasts express EphB receptors, and ephrin-B expression persists in the dermomyotome along their migratory pathway at the time of melanoblast migration (Santiago and Erickson, 2002). Disruption of EphB-ephrin-B interaction *in vivo* just

prior to the second wave of NCC migration prevents the melanoblasts from entering the dermomyotome, which suggests loss of an attractive or permissive signal. Indeed whereas chemotaxis of early NCCs *in vitro* is inhibited by the presence of ephrin-B1-Fc in a chemotaxis chamber, melanoblast chemotaxis is increased in response to ephrin-B1-Fc (Santiago and Erickson, 2002).

1.4.3 Rhombomere development and cranial neural crest migration.

1.4.3.1. Rhombomere formation.

During embryogenesis the developing hindbrain becomes segmented into repeating morphological compartments called rhombomeres. These compartments are not only morphologically discrete, but also have distinct genetic identity with sharp boundaries of gene expression corresponding to morphological boundaries (Lumsden and Krumlauf, 1996). The segmental pattern of cranial nerves is determined by the hindbrain organisation since cranial NCCs arising from particular rhombomeres migrate along specific pathways to the branchial arches where they contribute to specific ganglia (see section 1.4.3.2). Once the rhombomeres have formed there is a restriction of cell intermingling between neighbouring segments, although cells from different even numbered rhombomeres can mix, as can cells from odd numbered rhombomeres (Guthrie et al., 1993). A distinct morphological boundary will always form between cells of even origin and cells of odd origin (Guthrie and Lumsden, 1991).

There is complimentary expression of Eph receptors and ephrins on adjacent rhombomeres. Rhombomeres r3 and r5 express high levels of EphA4, EphB2 and EphB3, whereas the rhombomeres either side, r2, r4 and r6, express the ephrin-B ligands for these receptors (Becker et al., 1994; Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996a; Henkemeyer et al., 1994; Nieto et al., 1992) and Eph receptor-ephrin interaction at the rhombomere boundaries maintains the discrete compartments by restricting cell intermingling.

Mellitzer and colleagues have demonstrated restriction of cell intermingling by Eph receptors and ephrins appropriate to the hindbrain using a “fishball” assay. Mixing two cell populations from zebrafish animal caps each injected with a fluorescent tracer results in significant intermingling of the two populations, but this

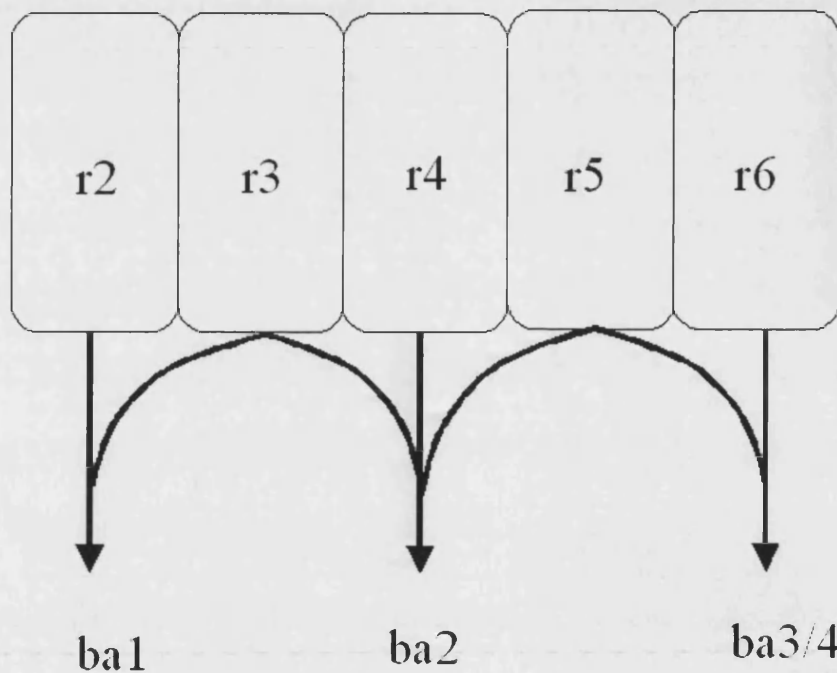
intermingling is restricted by the expression of EphA4 or EphB2 in one population and ephrin-B2 in the adjacent population (Mellitzer et al., 1999). A morphological boundary is seen between the cell populations, and, as in the hindbrain, this boundary corresponds to a region of reduced gap junction communication, thought to prevent the spread of regional identity signals between rhombomeres *in vivo* (Mellitzer et al., 1999). In the fishball assay bi-directional signalling is necessary for the restriction of cell intermingling since juxtaposition of truncated ephrin-B2 with full length EphB2, or vice versa, does not prevent cell mixing. Unidirectional signalling by either the Eph receptor or the ephrin is sufficient to prevent communication via gap junctions (Mellitzer et al., 1999).

Injection of dominant negative EphA4 into one cell of a 2 cell stage zebrafish or *Xenopus* embryo leads to disruption of the ordered rhombomeres and the presence of ectopic cells with r3/r5 identity within even numbered rhombomeres (Xu et al., 1995). Since ectopic ephrin-B2-positive cells sort to the boundaries of r3 and r5, but remain throughout even numbered rhombomeres it would appear that Eph receptors control the restriction of cell intermingling between odd and even fated rhombomeric cells, as opposed to influencing cell fate switching (Xu et al., 1999). This idea is supported by time-lapse microscopy of the movement of ectopic ephrin-B2 positive cells in the hindbrain. Although cells expressing truncated ephrin-B2 behave the same way as those expressing full length ephrin-B2, there is a contribution of ephrin-B reverse signalling to cell sorting, as truncated EphA4 expressed in a mosaic pattern leads to these cells being sorted to the edges of inappropriate (ephrin-B expressing) rhombomeres.

1.4.3.2.Cranial neural crest migration.

The NCCs of the hindbrain migrate into the branchial arches along specific pathways according to their rhombomeric origin, where they give rise to the facial cartilage and the bones and muscles of the neck as well as the cranial nerve ganglia. In chick, mouse and *Xenopus* NCCs from r2 migrate to the 1st branchial arch, NCCs from r4 will populate the 2nd branchial arch and NCCs from r6 migrate to the 3rd and 4th branchial arches. NCCs arising from r3 and r5 do not migrate through their adjacent mesoderm but join the streams migrating either side of them (Kulesa et al., 2004) (Fig 1.7). These migratory streams of NCCs remain separate. Despite subtle

Figure 1.7. Cranial NCCs migrate from rhombomeres to the branchial arches along distinct pathways during development.



Cranial neural crest cells (NCCs) migrate in separate streams from specific rhombomeres (r) to populate the branchial arches (ba). In chick and mouse cranial NCCs from r2 populate ba1, r4 gives rise to NCCs that populate ba2 and r6 NCCs invade ba3 and ba4. In *Xenopus* r6 NCCs populate ba4 and ba5. NCCs from r3 and r5 migrate with the adjacent rostral or caudal stream.

differences in the migratory pathway, destination and receptor-ligand expression of NCCs between species, studies in mice and *Xenopus* demonstrate a role for repulsive signals mediated by Eph receptor and ephrins in the guidance of cranial NCCs.

Complementary expression of Eph receptors and ephrins in neighbouring populations of cranial NCCs restricts intermingling between adjacent but differently fated NCC migratory streams. For example, in *Xenopus*, ephrin-B2 is expressed in the 2nd stream and EphA4 and EphB1 (both of which are receptors for ephrin-B2) are expressed in the 3rd stream (Smith et al., 1997). Dominant-negative versions of either

receptor causes Eph receptor-expressing crest normally destined for the 3rd arch to migrate aberrantly along with the more anterior stream of ephrin-B2-positive NCCs. This suggests that directional repulsive signalling between Eph receptor and ephrin expressing NCCs normally keeps the migrating streams separate (Smith et al., 1997). EphA-ephrin-A signalling also has a role in maintaining separate streams. In *Xenopus* NCCs destined for the 3rd branchial arch express EphA2. Introduction of dominant negative EphA2 allows intermingling of the 3rd and 4th branchial arch streams (Helbling et al., 1998).

Eph receptors and their respective ligands are expressed along the presumptive pathways taken by migrating cranial NCCs, and within the arches themselves, and in addition to restricting intermingling between migratory streams, Eph receptor-ephrin interaction prevents NCCs from invading inappropriate territory. In addition to a failure of NCCs to migrate in defined streams in ephrin-B2 knockout mice, NCCs invade territory that, in a wild-type animal would be ephrin-B2-positive, and thus from which they would normally be excluded. This repulsive exclusion is mediated by Eph receptor forward signalling since a knock in of ephrin-B2 without its cytoplasmic tail rescues the defects in NCC migration (Adams et al., 2001). It is also possible that Eph-ephrin signals are involved in patterning the branchial arches themselves as ephrin-B2 knockout mice have defective 2nd branchial arches (Adams et al., 2001).

1.5. Eph receptor signalling.

The biological functions of Eph receptors and ephrins described above all involve cell movements and/or changes in cell shape, which depend on co-ordinated changes in the organisation of the cell cytoskeleton and cellular adhesion. In molecular terms the intracellular signalling cascades induced by activation of Eph receptors ultimately influence the actin cytoskeleton and cell-matrix adhesions, to influence cell morphology and motility in a variety of *in vitro* systems.

1.5.1. Eph Receptor signalling to the actin cytoskeleton.

Activation of endogenous Eph receptors in a variety of cell types leads to reorganisation of filamentous actin (F-actin). These F-actin rearrangements induced by Eph receptor activation include the formation of contractile bundles of actin

filaments, known as stress fibres, and the retraction of cell processes (Lawrenson et al., 2002; Miao et al., 2003; Ogita et al., 2003) as well as protrusive actin structures such as membrane ruffles and filopodia (Nagashima et al., 2002; Penzes et al., 2003). As discussed in section 1.3, activation of neuronal EphA receptors cause growth cone collapse in a variety of neuronal cell types, a process that also involves regulation of the actin cytoskeleton (Meima et al., 1997a; Meima et al., 1997b).

Eph receptor-mediated control of actin rearrangements can occur through members of the Rho family of monomeric GTPases. The Rho GTPases cycle between an inactive, GDP-bound conformation, and an active GTP-bound conformation in which they bind to effector molecules and transduce intracellular signals. GTPase exchange factors (GEFs) act to increase the level of GTP-bound, active Rho family member by binding the inactive form of the GTPase and catalysing the exchange of GDP for GTP (Nobes and Hall, 1994; Quilliam et al., 1995). Conversely GTPase activating proteins (GAPs) catalyse the hydrolysis of bound GTP to GDP, attenuating signalling through the GTPase (Lamarche and Hall, 1994). The inactive, GDP-bound GTPase can be stabilised by binding to guanine nucleotide dissociation inhibitors (GDIs) (Olofsson, 1999). Regulation of GTPase activity is involved in many of the biologically relevant responses to Eph receptor activation such as the repulsive response of neuronal growth cones, the morphology of dendritic spines and changes in vascular smooth muscle cell contractility (Irie and Yamaguchi, 2002; Journey et al., 2002; Ogita et al., 2003; Penzes et al., 2003; Wahl et al., 2000). In addition recent evidence suggests that Eph receptors interact with members of the Abl family of non-receptor tyrosine kinases (Yu et al., 2001), which have also been implicated in the organisation of the actin cytoskeleton in neural and non-neural tissues (Van Etten, 1999)

1.5.1.1. Rho Family GTPases; regulators of the actin cytoskeleton.

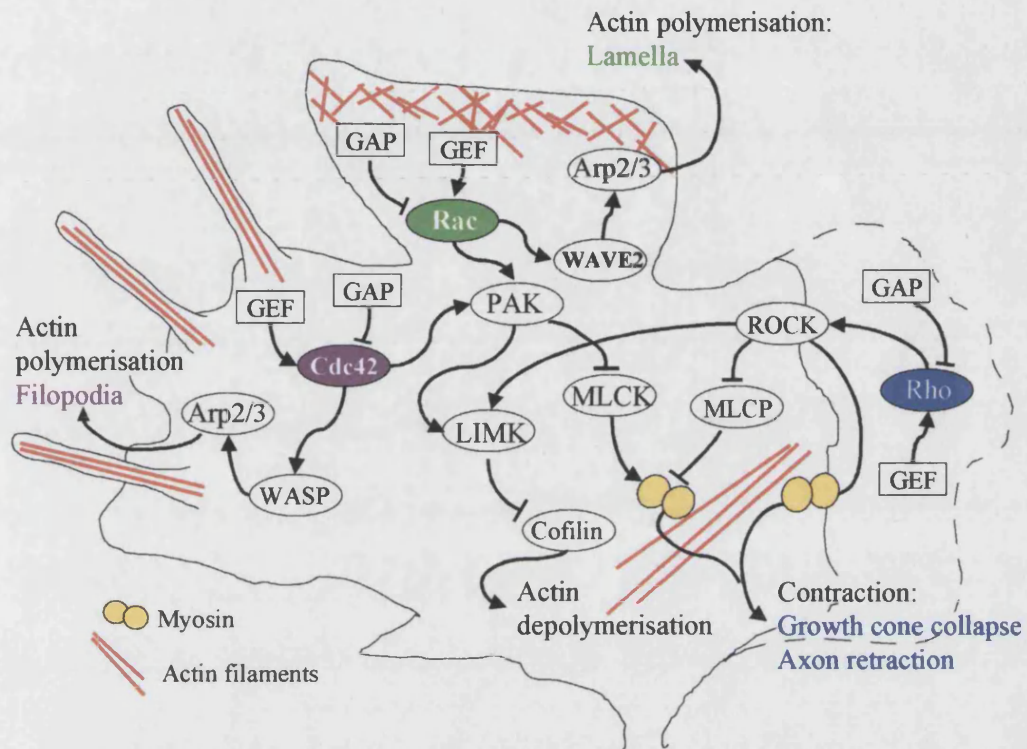
Despite the accumulating evidence that Rho family GTPases influence multiple intracellular signalling pathways, these proteins are best known for their pivotal role in regulating the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Classic experiments in fibroblasts have revealed that of the three members that exemplify the Rho GTPase family, Rac1 activity results in localised actin polymerisation at the cell periphery resulting in the formation of lamella, activation of Cdc42 stimulates

filopodial formation, and RhoA regulates cell contractility by inducing the formation of actomyosin cables known as stress fibres (Nobes and Hall, 1995; Ridley et al., 1992; Ridley, 1999). Similarly injection of mutant Rho family GTPases into neuronal cells reveals that Cdc42 activation leads to increased filopodial formation and Rac-dependent lamella at the growth cone, whereas RhoA activity results in growth cone collapse and neurite retraction (Kozma et al., 1997).*

The relevant cellular effector of RhoA during neurite retraction is likely to be Rho kinase (ROCK), since expression of a constitutively active form of ROCK is sufficient to cause neurite retraction in neuroblastoma cells *in vitro* (Amano et al., 1998). ROCK binds to, and is activated by, RhoA-GTP (Leung et al., 1995; Matsui et al., 1996) and active ROCK can phosphorylate the regulatory light chain of myosin II (myosin light chain, MLC), increasing myosin ATPase activity (Amano et al., 1996). In addition to this direct increase in MLC phosphorylation, active ROCK phosphorylates and inhibits myosin light chain phosphatase (Kimura et al., 1996) which would otherwise remove ROCK-phosphorylated residues on MLC.* Phosphorylation of MLC leads to the assembly of contractile actin-myosin bundles known as stress fibres (Citi and Kendrick-Jones, 1987). In this way Rho activation leads to an increase in cell contractility via ROCK (Riento and Ridley, 2003). Rho- and ROCK-induced neurite retraction probably acts via increased actomyosin contraction, since a mutant version of MLC, which alone increases the activation of myosin ATPase, promotes neurite retraction *in vitro* (Amano et al., 1998).

Given that Rac1 and Cdc42 activation gives rise to the protrusive actin structures of lamella and filopodia respectively it is plausible to expect there to be a link between the activity of these GTPases and actin polymerisation machinery. Actin related proteins Arp2 and Arp3, and the WASP/WAVE family of actin nucleation factors define an actin assembly pathway that can be influenced by both Rac and Cdc42 (Higgs and Pollard, 2001). The Arp2/3 complex binds the side of an existing actin filament and nucleates polymerisation of an actin side branch (Amann and Pollard, 2001; Blanchoin et al., 2000). Members of the WASP/WAVE family of proteins bind the Arp2/3 complex and stabilise an active Arp2/3 conformation to promote nucleation (Amann and Pollard, 2001; Higgs and Pollard, 2001). Active Cdc42 can bind the regulatory domain of WASP, disrupting an inhibitory intramolecular interaction and allowing WASP to activate the Arp2/3 complex (Rohatgi et al.,

Fig 1.8. Regulation of actin dynamics in the growth cone by Rho family GTPases.



GTPase-mediated signalling pathways are regulated by GEFs (Guanine nucleotide exchange factors), which increase the level of active, GTP-bound GTPase, and GAPs (GTPase activating proteins), which catalyse the hydrolysis of GTP for GDP. The GTPases Cdc42 and Rac promote actin polymerisation via the WASP/WAVE family of actin nucleation factors and the Arp2/3 complex to stimulate the formation of filopodia (Cdc42) and lamellae (Rac). Cdc42 and Rac also share a common effector in PAK, which inhibits the activity of myosin light chain kinase (MLCK) to reduce actomyosin contractility, and activate LIM kinase (LIMK) to inhibit the actin depolymerisation factor cofilin, thus stabilising actin filaments. Rho acts via its effector ROCK to directly phosphorylate the regulatory light chain of myosin, and also inhibit myosin light chain phosphatase, thereby increasing contractility, leading to growth cone collapse and axon retraction. ROCK can also activate LIMK.

1999). Rac1 can stimulate actin polymerisation by activating the Arp2/3 complex via WAVE2 (Takenawa and Miki, 2001), possibly by inducing the relocalisation of a protein complex including WAVE2 within the cell (Steffen et al., 2004).*

Rac1 and Cdc42 have a common effector in p21-activated kinase (PAK) (Aspenstrom, 1999). Rac and Cdc42 activation leads to an increase in PAK activity, which in turn regulates the activity of other cellular kinases involved in actin rearrangement. PAK phosphorylation of LIM kinase increases its activity towards the actin depolymerising factor cofilin (Dan et al., 2001). Since cofilin is negatively regulated by phosphorylation, PAK activation of LIM kinase ultimately leads to the stabilisation of actin filaments. In addition, PAK-mediated phosphorylation of myosin light chain kinase (MLCK) reduces its ability to phosphorylate myosin, and hence PAK activity can reduce actomyosin based contraction (Wirth et al., 2003). In this way Cdc42 and Rac activation promote protrusive actin structures by stimulation of actin polymerisation and stabilising existing actin filaments, and antagonise the actomyosin contraction that underlies Rho-mediated changes in the actin cytoskeleton.*

* See Fig. 1.8

1.5.1.2. Eph receptor regulation of Rho GTPases.

Given that Rac and Cdc42 activity stimulates protrusive actin structures, while Rho activity increases cell contractility, an overall model might predict that the repulsive responses induced by Eph receptor-ephrin interaction during development, for example in axon and neural crest guidance, might act through the Rho pathway rather than Rac and Cdc42. Activation of endogenous Eph receptors has been shown to increase the level of RhoA-GTP in a number of non-neuronal cell types, without a corresponding change in the activity of Rac1 or Cdc42 (Lawrenson et al., 2002; Miao et al., 2003; Ogita et al., 2003; Sharfe et al., 2002). Rho activity is important for the cellular response to ephrin stimulation, since blocking ephrin-induced increase in Rho activity prevents ephrin-induced retraction of melanoma cell line processes, and the formation of stress fibres in smooth muscle cells (Lawrenson et al., 2002; Ogita et al., 2003). Ephrin-A stimulation of RGCs in culture also stimulates RhoA activity, and inhibiting Rho activity with C3 toxin prevents ephrin-A-induced growth cone collapse (Wahl et al., 2000; see section 1.5.3).

Eph receptor-mediated increases in RhoA activity have been shown to involve the activity of Rho GEFs, which play an important role in mediating biologically relevant responses to Eph receptor activation. For example the Rho GEF Ephexin mediates ephrin-A-induced RGC growth cone collapse *in vitro* (Shamah et al., 2001; see section 1.5.3) and the structurally related VSMRhoGEF (vascular smooth muscle Rho GEF) regulates Rho dependent contractile actin structures in smooth muscle cells (Ogita et al., 2003).

Vascular smooth muscle cell contractility is important for the maintenance of normal blood circulation, and this contractility is regulated by the RhoA-ROCK-myosinII pathway described in section 1.5.1.1 (Somlyo and Somlyo, 2000). The *in vivo* expression pattern of VSMRhoGEF in smooth muscle of many organs matches that of EphA4, and co-expression of EphA4 and VSMRhoGEF in smooth muscle cells leads to the specific activation of RhoA (Ogita et al., 2003). Reducing VSMRhoGEF expression by RNAi prevents ephrin-A-induced Rho activation and associated increase in contractile stress fibres in these cells (Ogita et al., 2003). Although the result of EphA4 activation on VSMRhoGEF activity was not investigated, these observations suggest that Eph-receptor-mediated phosphorylation of VSMRhoGEF increases Rho activity and hence regulates vascular smooth muscle cell contractility. This is interesting given that ephrin-Eph receptor interaction is required for normal development of vascular smooth muscle (Zhang et al., 2001), and suggests that Eph receptor signalling in vascular smooth muscle cells may act not only to regulate cell behaviour during development, but also during the normal physiological function in the adult.

In addition to the above evidence that Eph receptors can activate Rho via GEFs, there is also evidence that Eph receptor signalling can act via Rac and Cdc42 to induce rearrangements of the actin cytoskeleton that result in the protrusion of actin-rich structures. For example the formation of dendritic spines involves remodelling of filopodial protrusions into longer actin structures, a process that involves direct regulation of actin polymerisation (Halpain, 2000). Over-expression of EphB2 in hippocampal neurons, or stimulation of these neurons with ephrin-B1, induces the formation of dendritic spines (Ethell et al., 2001; Penzes et al., 2003), and recent investigations have revealed a role for both Cdc42 and Rac activity in this process,

mediated by the Rho family GEFs Intersectin and Kalirin respectively (Ethell et al., 2001; Irie and Yamaguchi, 2002; Penzes et al., 2001; Penzes et al., 2003).

Stimulation of primary hippocampal neurons with ephrin-B1 results in increased levels of active Cdc42, and this is the result of a functional interaction between EphB2 and the Cdc42-specific GEF Intersectin (Irie and Yamaguchi, 2002). Endogenous neuronal EphB2 interacts with Intersectin, and co-expression of the two proteins increases Intersectin GEF activity towards Cdc42 (Irie and Yamaguchi, 2002). Expression of a kinase-dead EphB2, or a truncated form of Intersectin lacking the GEF domain, blocks the formation of dendritic spines that normally appear on primary hippocampal neurons after a few weeks in culture (Ethell et al., 2001; Irie and Yamaguchi, 2002), suggesting that EphB receptor activation normally acts via Intersectin and Cdc42 to initiate and remodel dendritic spines during development.

A second Rho family GEF that has been implicated in ephrin-mediated changes in dendritic spine morphology is Kalirin, a member of the Trio family of GEFs. Over-expression of Kalirin in hippocampal neurons results in an increase in size and number of dendritic spines, qualitatively similar to the effect of ephrin-B1 stimulation of hippocampal neurons *in vitro* (Penzes et al., 2001; Penzes et al., 2003). The changes in spine morphology are both Eph kinase- and Rac-dependent (Penzes et al., 2003), but although Kalirin exhibits *in vitro* GEF activity for Rac, and Kalirin GEF activity is required for ephrin-B1-induced changes in dendritic spine morphology, EphB2 activation does not change Kalirin's GEF activity towards Rac (Penzes et al., 2000; Penzes et al., 2003). In agreement with this observation no increase in Rac activity is seen in hippocampal neurons in response to ephrin-B1 stimulation (Ethell et al., 2001). Instead, activation of EphB2 receptors on dendritic spines leads to a redistribution of endogenous Kalirin to synaptic clusters (Penzes et al., 2003). This adds an extra level of subtlety to the capacity of Eph receptors to induce highly localised changes in the actin cytoskeleton via modulation of the Rho GTPases. As well as activating GEF activity in a spatially discrete region, at or near the site of Eph receptor activation, Eph receptor activation could increase the concentration of previously active GEF to a very precise location, for example the dendritic spine.

Overall the experiments described above provide evidence that Eph receptor signalling can induce physiologically relevant changes in the actin cytoskeleton via

Rho family GTPases and their GEFs, and that the cellular responses invoked by Eph-receptor activation can be both repulsive/retractive, via activation of Rho, or attractive/protrusive via Rac or Cdc42 activity. It should be noted however that the Rac/Cdc42 attraction and Rho repulsion pattern is not without notable exceptions. Rac activity is required to mediate cell-cell separation events during EphB-mediated repulsive responses, and is also necessary for EphA-mediated axon repulsion (Jurney et al., 2002; Marston et al., 2003). It is likely that since Eph receptor-ephrin interaction mediates such a complex array of morphogenic processes *in vivo*, Eph receptor activity regulates many intracellular signalling intermediates, including members of the Rho family, in a tightly controlled and co-ordinated manner.

1.5.1.3. Eph receptor signalling and Abl family kinases.

Members of the Abl family of non-receptor tyrosine kinases have been implicated in rearrangement of the actin cytoskeleton in response to signals mediated by many cell surface receptors (Hernandez et al., 2004; Woodring et al., 2003) as well as the control of axon guidance during development (Lanier and Gertler, 2000; Moresco and Koleske, 2003). Biochemical interactions between Abl and EphB2 have been reported, and these interactions are conserved between EphB2 and the Abl homologue, Arg (Abl related gene) (Yu et al., 2001). EphB2 association with the SH2 domain of Abl and Arg kinases depends on Eph kinase activity, and phosphorylation of the two conserved tyrosine residues in the Eph receptor juxtamembrane region (Yu et al., 2001). Co-expression of full length EphB2 results in phosphorylation of Abl and Arg, and this is also dependent on Eph kinase activity, although it is not clear whether Abl and Arg are directly phosphorylated by Eph kinases (Yu et al., 2001). Stimulation of cells expressing endogenous EphB2 with ephrin-B1 leads to a transient decrease in Abl kinase activity (Yu et al., 2001), but despite the intriguing possibility that Eph receptors may phosphorylate and regulate Abl kinase in response to physiological signals, no functional role for Abl activity during Eph receptor-mediated cell behaviour has been shown to date.

1.5.2. Eph receptor signalling and adhesion.

Cell attachment to the extracellular matrix (ECM) is mediated by integrins, which are transmembrane heterodimers that link the ECM to the cytoskeleton. Engagement of integrins by ECM proteins results in the recruitment of structural and signalling

proteins at sites of cell-matrix adhesion, and ECM activation of integrin signalling induces changes in cytoskeletal organisation; so called “outside-in signalling” (Humphries, 2000). Integrin-mediated adhesion can be influenced by intracellular signals, which either induce a conformational change in the integrin heterodimers, regulating their affinity for ligands, or regulate integrin clustering, changing their avidity (Hughes and Pfaff, 1998). This “inside-out” signalling is not well understood but there is evidence that the intracellular signalling pathways stimulated by Eph receptor activation may modulate integrin-mediated adhesion.

Activation of the small GTPase R-Ras increases cell adhesion to ECM proteins by directly regulating integrin affinity and avidity, and inhibition of R-Ras prevents cells from maintaining integrin-mediated attachment (Kinashi et al., 2000; Kwong et al., 2003; Zhang et al., 1996b). The reported ability of EphB receptors to modulate R-Ras activity correlates with EphB-dependent changes in cell-matrix adhesion. EphB2 activation, which results in loss of cell-matrix adhesion in epithelial cells, leads to the phosphorylation of R-Ras effector domain, preventing effector binding (Zou et al., 1999) and thus inhibiting downstream signalling. Expression of a non-phosphorylatable mutant of R-Ras prevents EphB2-mediated loss of attachment, suggesting that EphB2 activity acts to reduce cell matrix adhesion by phosphorylating R-Ras (Zou et al., 1999). The R-Ras/EphB2 interaction may depend on the adaptor SHEP1, which associates with EphB2 in an activity dependent manner, and also binds R-Ras (Dodelet et al., 1999).

In contrast to EphB2, other studies have shown that EphB1 activation increases integrin-mediated adhesion in a variety of cell types (Becker et al., 2000; Huynh-Do et al., 1999; Stein et al., 1998b). EphB1 activation induces the formation of a protein complex that includes RasGAP and phosphorylated p62DOK, a protein which, when phosphorylated, can bind to and inactivate RasGAP (Becker et al., 2000; Kashige et al., 2000). Since RasGAP can downregulate R-Ras (Rey et al., 1994) and R-Ras activity can be correlated with an increased cell-matrix adhesion (Kinashi et al., 2000; Kwong et al., 2003; Zhang et al., 1996b) it is therefore possible that EphB1-mediated phosphorylation of p62DOK inhibits RasGAP, and the resulting increase in R-Ras activity leads to increased cell-matrix adhesion. It is interesting to note however that activation of EphB2, which as discussed above can increase adhesion, can also lead to phosphorylation of p62DOK (Becker et al., 2000). This suggests that

differential regulation of R-Ras by p62DOK phosphorylation cannot be the only mechanism underlying the opposing effects of EphB1 and EphB2 on integrin-mediated adhesion.

During inside-out signalling, phosphorylation of focal adhesion kinase (FAK) correlates with increased cell-matrix adhesion, and is thought to be required for R-Ras-induced increases in adhesion (Kwong et al., 2003; Pichard et al., 2001). Dephosphorylation of FAK following EphA2 activation correlates with ephrin-A1-induced loss of integrin-mediated adhesion (Miao et al., 2000). The Phosphatase SHP2 is recruited to EphA2 following stimulation with ephrin-A1, and expression of a dominant negative SHP2 leads to elevated levels of phosphorylated (Miao et al., 2000). Dominant negative SH2 also inhibits ephrin-A1-induced loss of adhesion, suggesting that EphA2-mediated activation of SHP2 normally reduces adhesion by the dephosphorylation of FAK (Miao et al., 2000).

Recently it has been shown that PI3 kinase may be involved in phosphorylation of FAK during the regulation of cell adhesion by R-Ras (Kwong et al., 2003). Interestingly EphA8 mediated increases in cell adhesion depend on PI3 kinase activity, providing an additional link between Eph receptor signalling and regulation of FAK-mediated cell-matrix adhesions (Gu and Park, 2001).

Overall the experimental data suggests that Eph receptors have the potential to activate R-Ras via p62DOK-mediated inhibition of RasGAP, increasing integrin-

mediated attachment through FAK and possibly PI3 kinase. Alternatively Eph receptor activation could directly phosphorylate R-Ras and inhibit a pathway that leads to FAK phosphorylation, and/or recruit SHP2 to dephosphorylate FAK, reducing integrin-mediated adhesion. The capacity of Eph receptors to both increase and decrease a signalling pathway is not restricted to R-Ras however. Unlike R-Ras, the closely related H-Ras signals through the MEK/ERK pathway, and there are reports of Eph receptor activation both positively and negatively regulating MEK and ERK (Elowe et al., 2001; Miao et al., 2001; Pratt and Kinch, 2002; Pratt and Kinch, 2003; Vindis et al., 2003; Zisch et al., 2000). H-Ras can modulate integrin adhesions via MEK/ERK (Hughes et al., 1997) and there is evidence that EphB-mediated regulation of the MEK/ERK pathway plays a role in ephrin-B-induced cell migration (Vindis et al., 2003) and neurite retraction (Elowe et al., 2001; see section 1.5.3), but to date there is no direct evidence that the MEK/ERK pathway mediates these cellular effects via changes in cell-matrix adhesion (Vindis et al., 2003).

To date Eph receptor-dependent activation or repression of R-Ras signalling is the only factor that correlates with Eph receptor-mediated increase or decrease in cell-matrix adhesion. There does not appear to be a correlation between the adhesion effects and the subclass of Eph receptor that is active, or the method of ligand presentation (soluble vs. substrate bound). It is interesting to note that the majority of experiments performed to determine the effect of Eph receptor signalling on cell adhesion have utilised cell lines expressing abnormally high levels of Eph receptors, either as a consequence of transfection during an experimental protocol, or transformation prior to immortalisation. In either case the intracellular machinery recruited by active Eph receptors under normal physiological conditions might be absent or abnormally regulated. It would be interesting to address the effect of Eph receptor activation on adhesion of a cell type which endogenously expresses Eph receptor, and to which changes in adhesion are physiologically relevant, for example during neural crest migration or repulsive axon guidance.

1.5.2.1. Eph receptor signalling and Src family kinases.

The Src family of non-receptor tyrosine kinases can be activated by many different classes of cellular receptor, including growth factor, chemokine and adhesion receptors, and are involved in many biological activities, including regulation of the

actin cytoskeleton and cell matrix adhesions (Frame et al., 2002; Thomas and Brugge, 1997). Of particular interest in terms of Eph receptor signalling is the evidence that Src kinase activity is necessary for cell motility, namely that fibroblasts deficient in Src family kinases show impaired migration *in vitro* (Fincham and Frame, 1998; Hall et al., 1996; Klinghoffer et al., 1999), and recently Src kinase activity has been implicated in mediating cell migration induced by Eph receptor activation (Steinle et al., 2002; Vindis et al., 2003).

Several Eph receptors, including EphB1 and EphB2, have been shown to interact with Src, and EphA3, EphA4, EphA8, and EphB3 can associate with the closely related kinase Fyn (Choi and Park, 1999; Ellis et al., 1996; Hock et al., 1998a; Prevost et al., 2002). In all cases investigated the interaction with Eph receptor depends on the SH2 domain of the Src family member in question, and the observation that substitution of the juxtamembrane tyrosine residues with phenylalanine abrogates Src or Fyn binding has been interpreted as evidence that Src and Fyn bind specifically to the phosphorylated juxtamembrane region. As mentioned in section 1.2.3 recent evidence that these residues mediate an autoinhibitory interaction suggests that such results must be interpreted with care, since phenylalanine substitutions will result in a receptor that is permanently in an inactive conformation and may therefore have reduced accessibility to many binding sites. However the motifs surrounding the juxtamembrane tyrosines correspond to high affinity Src binding sites (Ellis et al., 1996; Songyang et al., 1993), which supports the idea that the Src kinases bind in this region. Importantly activation of Eph receptors has been shown to increase the activity of Src and/or Fyn as assayed by an increased level of autophosphorylation (Sharfe et al., 2003; Steinle et al., 2002; Takasu et al., 2002; Vindis et al., 2003).

As discussed in section 1.4.1 EphB4-ephrin-B2 interaction is crucial for remodelling of the primary vascular plexus during embryogenesis, and directed cell movements underlie this *in vivo* remodelling and the *in vitro* sprouting angiogenesis induced in endothelial cells in culture following EphB4 stimulation (Adams et al., 1999; Adams et al., 2001; Gerety et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998). Primary human microvascular endothelial cells, which endogenously express EphB4, show a positive chemotactic response to ephrin-B2, and this response is blocked by inhibiting Src kinase activity (Steinle et al., 2002). Similarly ephrin-B2 stimulation

of a cell line that endogenously expresses EphB1 leads to a Src-dependent chemotactic response (Vindis et al., 2003). In both cell types Src becomes phosphorylated on tyrosine 416, which is indicative of increased Src kinase activity (Kmieciak et al., 1988; Steinle et al., 2002; Vindis et al., 2003).

EphB1- and EphB2-mediated migration of these cells is also dependent on the activities of MEK and PI3 kinase respectively, and Src activity is required for the activation of both these pathways downstream of Eph receptor activation (Steinle et al., 2002; Vindis et al., 2003). It is interesting that both MEK and PI3 kinase are potentially involved in ephrin-mediated changes in cell-matrix adhesion, as discussed above. Given the accumulating evidence that Src regulates the turnover of cell-matrix adhesions, and that Src's role in disassembling adhesions is required for normal cellular migration (Carragher et al., 2001; Fincham and Frame, 1998; Hall et al., 1996; Klinghoffer et al., 1999; Webb et al., 2004) it is possible that Eph receptor-mediated increases in Src activity might contribute to ephrin-induced changes in adhesion, and hence allow successful migration in response to ephrin. Addition of a MEK inhibitor, or expression of a dominant negative MEK prevents Src-mediated disassembly of cell-matrix adhesions (Carragher et al., 2003). Since MEK has been implicated in Eph receptor-mediated changes in adhesion, and ephrin-induced growth cone collapse (see section 1.5.3) it is possible that Src activity plays a functional role downstream of Eph receptor signalling during axon guidance. It has been reported that ephrin-B2-mediated increase in cell adhesion is not affected by inhibiting Src activity (Vindis et al., 2003). However as discussed above Eph receptor activation can either increase or decrease cell-matrix adhesion, and Src activity is thought to regulate cell adhesion by specifically disassembling adhesion complexes (Carragher et al., 2001; Fincham et al., 1995; Fincham and Frame, 1998; Webb et al., 2004). It is therefore possible that Src activity is required during Eph receptor-induced loss of adhesion, for example during growth cone collapse.

1.5.3. Eph Receptor signalling and axon guidance.

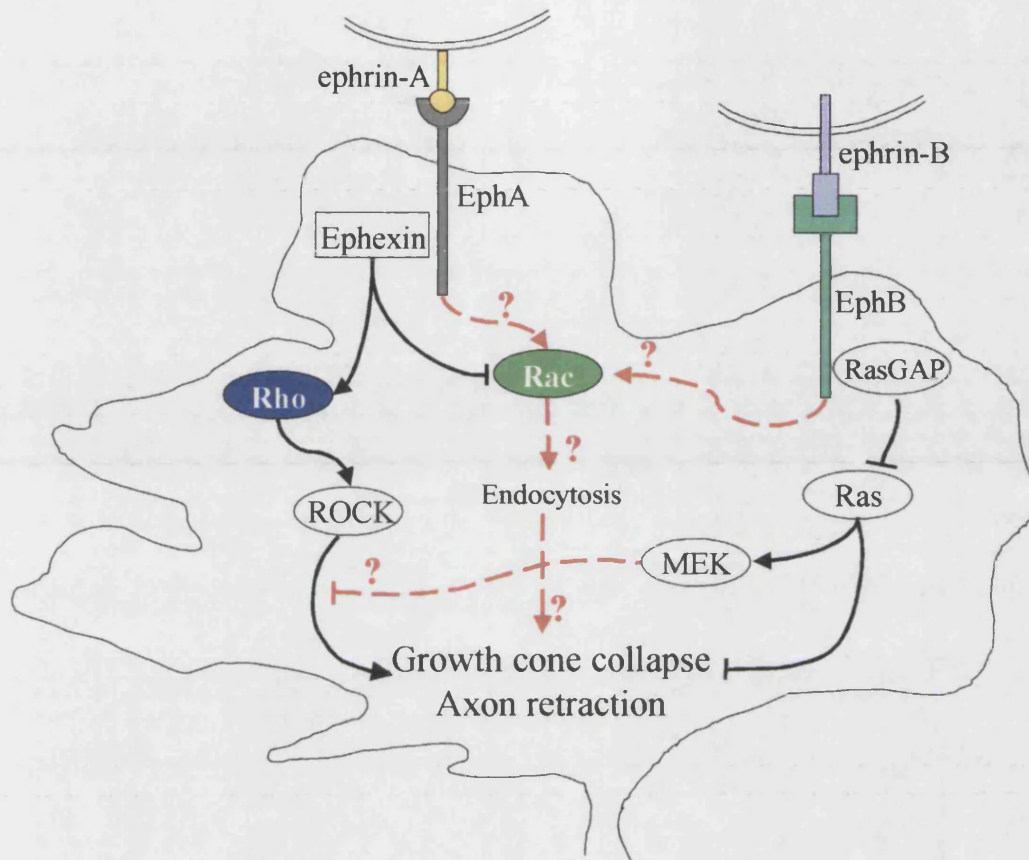
As discussed in section 1.3 the ephrins act as repulsive guidance cues for many classes of axon *in vivo*. Stimulation of these neurons with soluble ephrin *in vitro* results in loss of the F-actin-rich lamella and filopodia of the growth cone, which reflects ephrin-induced rearrangements of the actin cytoskeleton (Cheng et al., 2003;

Fournier et al., 2000; Journey et al., 2002; Meima et al., 1997a; Meima et al., 1997b; Menzel et al., 2001; Monschau et al., 1997; Shamah et al., 2001; Wahl et al., 2000). Early experiments showed that disrupting the actin cytoskeleton prevents axons responding to repulsive guidance cues (Bentley and Toroian-Raymond, 1986), and more recently it has been shown that integrin-mediated cell adhesions are rapidly disassembled in response to repulsive axon guidance cues (Barberis et al., 2004; Serini et al., 2003). In agreement with the idea that repulsive axon guidance cues coordinately regulate the actin cytoskeleton and cell-matrix adhesion, many of the Eph receptor signalling intermediates discussed above have a role in ephrin-induced growth cone collapse.

As discussed in section 1.5.1, modulation of Rho GTPase activity is a major mechanism whereby Eph receptor activation can influence the actin cytoskeleton. Stimulation of RGCs in culture with ephrin-A5-Fc leads to an increase in the levels of active Rho in the cells, and this increase is necessary for ephrin-induced growth cone collapse, since inhibition of Rho activity with C3 transferase significantly reduces the proportion of RGC growth cones that collapse in response to ephrin-A5 stimulation (Wahl et al., 2000). Inhibition of the Rho effector ROCK also inhibits ephrin-induced growth cone collapse (Wahl et al., 2000). EphA4, one of the Eph receptors endogenously expressed by RGCs *in vitro* and *in vivo*, has been found to associate with Ephexin, a GEF that has preferential activity for Rho over Cdc42 and Rac (Monschau et al., 1997; Shamah et al., 2001). The repulsive response of RGCs to ephrin requires Ephexin exchange activity, since expression of a dominant negative Ephexin reduces the ephrin-A5-induced collapse (Shamah et al., 2001). Together these results suggest Eph receptor activation leads to Ephexin-mediated increase in Rho activity, and growth cone collapse via ROCK.*

In addition to modulation of the Rho pathway, stimulation of RGCs with ephrin-A2 and ephrin-A5 has been shown to reduce the levels of active Rac (Journey et al., 2002; Wahl et al., 2000). Whereas ephrin-induced increase in Rho activity correlates well with the observation that activation of Rho is sufficient to cause neurite retraction (Kozma et al., 1997) the functional significance of the reduction of Rac activity in response to ephrin is not clear, since inhibition of Rac activity alone does not cause RGC growth cone collapse, and expression of a constitutively active Rac does not inhibit ephrin-induced growth cone collapse (Journey et al., 2002). The activities of

Fig 1.9. EphA and EphB receptor-mediated signalling pathways induce growth cone collapse and axon retraction.



EphA receptors constitutively associate with the GEF Ephexin, and when EphA receptors become activated, Ephexin increases the levels of active Rho, while decreasing the levels of active Rac. EphA-mediated growth cone collapse and axon retraction occurs through activation Rho, and its effector ROCK, which increase actomyosin-based contractility. EphB receptor activation induces growth cone collapse and axon retraction via RasGAP-mediated inhibition of the Ras-MEK pathway. The mechanism by which this occurs is unclear, although there is evidence that the Ras-MEK pathway may antagonise Rho-ROCK signalling in non-neuronal cells. Despite the transient decrease in Rac activation that follows EphA activation, Rac activity is required for ephrin-A-induced growth cone collapse and axon retraction, possibly for the internalisation of growth cone membrane.

Rho and Rac/Cdc42 have been suggested to be mutually antagonistic during growth cone responses to repulsive cues, including ephrins, and the simultaneous activation of Rho and inhibition of Rac following ephrin stimulation of RGCs might shift the balance away from Rac/Cdc42 actin filament assembly, and favour collapse (Giniger, 2002; Hirose et al., 1998; Kozma et al., 1997; Wahl et al., 2000). Expression of Ephexin in fibroblasts results in a mixed phenotype of Rho-, Rac- and Cdc42-dependent structures, namely stress fibres, lamellae and filopodial extension respectively (Shamah et al., 2001). Upon co-transfection of EphA4 and Ephexin however, the majority of cells exhibit Rho-like phenotypes at the expense of Rac- and Cdc42-like phenotypes, which might suggest that EphA receptor activity potentiates Ephexin GEF activity towards Rho, while reducing GEF activity towards Rac and Cdc42, although this was not tested directly (Shamah et al., 2001).

Stimulation of RGCs in culture with soluble ephrin-A5 and ephrin-A2 leads to an increase in endocytosis, assayed by uptake of fluorescent dextran from the bathing medium, and this ephrin-mediated endocytosis is dependent on Rac activity (Fournier et al., 2000; Journey et al., 2002). Recent work has shown that Rac dependent trans-endocytosis of EphB-ephrin-B complexes is necessary for separation of cells following receptor-ligand interaction at sites of cell-cell contact (Marston et al., 2003, Zimmer et al., 2003). This mechanism is thought to allow repulsive responses and cell-cell separation following an initial high affinity binding of Eph receptor and ligand (Marston et al., 2003). An alternative, Rac-independent, mechanism has been shown to mediate the separation of an EphA-expressing axon from an ephrin-A-expressing cell (Hattori et al., 2000). EphA3 binding stimulates proteolysis of ephrin-A2 by the metalloprotease ADAM10, and expression of a non-cleavable ephrin-A2 prevents axon withdrawal following contact with ephrin-A2 expressing cell (Hattori et al., 2000). Given that growth cone collapse is not inhibited by lack of ephrin-A2 cleavage (Hattori et al., 2000), it is possible that Rac-dependent endocytosis contributes to the loss of growth cone structures via internalisation of membrane (Journey et al., 2002) and that this explains the necessity for Rac activity during ephrin-A-induced growth cone collapse.*

In addition to effects on the Rho GTPases there is evidence that Eph receptors might influence axon guidance decisions via the Ras-MEK-ERK kinase pathway. Ephrin-B1-Fc stimulation of a neuronal cell line stably expressing EphB2 triggers the loss of

growth cone-like structures and neurite retraction, and induces a reduction in the phosphorylation levels of both ERK and MEK (Elowe et al., 2001). The responses are likely mediated by RasGAP activity, since expression of dominant negative RasGAP inhibits ephrin-B1-induced suppression of the MAP kinase pathway and constitutively active Ras blocks ephrin-B1-induced growth cone collapse and axon retraction (Elowe et al., 2001). Taken together this evidence suggests that ephrin-B-induced axon guidance may be mediated by activation of RasGAP and subsequent decrease in Ras and MEK/ERK activity. One common way in which tyrosine kinase receptors modulate Ras activity is via the adaptor Grb2 complexed with the Ras exchange factor SOS, which together activate the Ras-MEK-ERK pathway (Downward, 1996). No association is seen between EphB2 and Grb2 in neuronal cell lines used above, which correlates with EphB2-induced reduction in ERK/MEK phosphorylation (Elowe et al., 2001). Interestingly, ablating the RasGAP binding domain of EphB2 and simultaneously introducing a Grb2 binding site leads to upregulation of the MAP kinase pathway and abolishes neurite retraction following ephrin-B1-Fc stimulation (Tong et al., 2003). Given that EphB1 activation can activate ERK via recruitment of Grb2 in some cell types (Vindis et al., 2003) it would be interesting to determine whether the potential upregulation of Ras activity via Grb2 and SOS might underlie the attractive responses seen in some axons following EphB activation (Hindges et al., 2002; Mann et al., 2003).

The mechanism whereby Eph-mediated reduction in the Ras-MAP kinase pathway leads to growth cone collapse is not clear. Reduction in MEK/ERK activity correlates with increased integrin-mediated adhesion, rather than the reduction in adhesion which would be assumed to accompany growth cone collapse and axon retraction (Barberis et al., 2004; Hughes et al., 1997; Serini et al., 2003). Dominant negative H-Ras has been shown to reduce integrin-mediated adhesion in some cell types, but this is independent of the MEK/ERK pathway (Shibayama et al., 1999). However, stress fibre formation induced by active Rho or ROCK is compromised in cells expressing constitutively active Ras, and this effect is ameliorated by inhibition of MEK activity (Sahai et al., 2001). In addition a function-blocking anti-Ras antibody prevents neurite retraction in response to LPA (Leblanc et al., 1998). These results suggest that activation of the Ras-MEK-ERK pathway inhibits ROCK activity and provide an alternative mechanism by which Eph receptor signalling, via reduction of

the MAP kinase pathway, could stimulate ROCK-mediated growth cone collapse and axon retraction (See Fig. 1.9).

1.5.3.1. Modulation of Eph receptor-mediated growth cone collapse.

During recent years a great deal of interest has been stimulated by the observation that intracellular levels of cyclic nucleotides can modulate the response of *Xenopus* neurons to axonal guidance cues. For example the attractive response of an axon to Netrin can be switched to repulsion by reducing intracellular levels of cAMP in the neuron (Song and Poo, 2001). Although initial studies suggested that guidance cues can be placed into one of two groups depending on whether they are sensitive to levels of cAMP or cGMP, recent evidence suggests that the ratio of cAMP to cGMP can modulate the response to a single cue (Nishiyama et al., 2003; Song and Poo, 2001)

Xenopus RGCs collapse in response to ephrin-B1-Fc stimulation, and this repulsive response is reduced by inhibiting PKG activity, suggesting that EphB-induced growth cone collapse is mediated by high intracellular levels of cGMP (Mann et al., 2003). The collapse assay used in this study cannot address whether modulating cGMP levels can switch the repulsive response to ephrin-B1 to an attractive response. However use of the growth cone turning assay has revealed a role for ECM proteins in modulating the neuronal response to ephrin. When exposed to a gradient of soluble ephrin-A5 a subpopulation of *Xenopus* RGC axons growing on fibronectin turn away from the ephrin-A5 source, whereas neurons growing on laminin exhibit an attractive response, turning towards the source (Weinl et al., 2003). These observations highlight the incredible complexity that must underlie the integration of intracellular signalling pathways during axon guidance. Not only do Eph receptors have the potential to influence cytoskeletal dynamics and integrin mediated adhesion, but these signals are also influenced by intracellular signals mediated by many other classes of receptor.

1.6. Src family kinases.

Members of the Src family of non-receptor tyrosine kinases are key components of signal transduction pathways for a wide range of cellular processes including cell growth, migration and differentiation, which all require co-ordinated changes in the

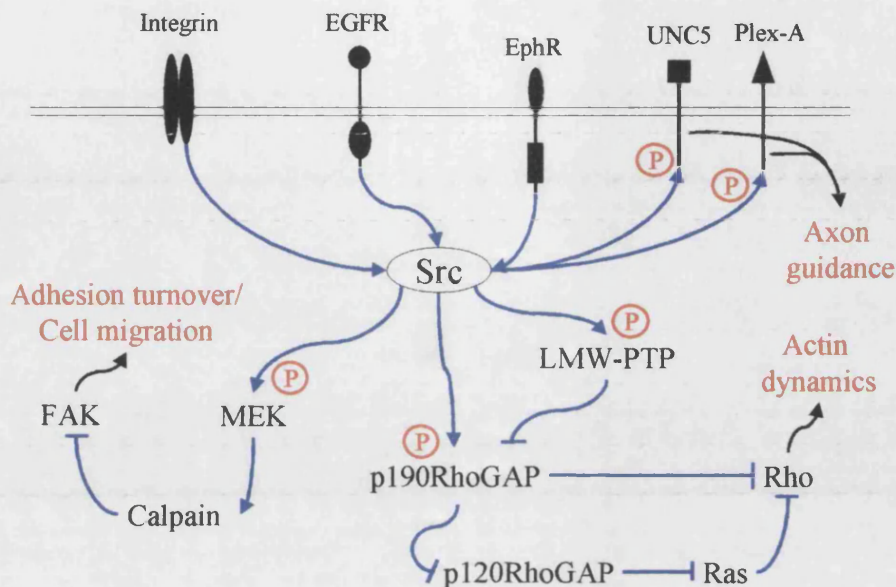
cellular adhesion and cytoskeletal machinery. For example an increase in Src activity and phosphorylation of Src substrates occurs in response to the activation of growth factor receptors, and Src is important for transducing signals initiated by these receptor tyrosine kinases to downstream targets, including components of the actin cytoskeletal machinery (Abram and Courtneidge, 2000; Thomas and Brugge, 1997). Of the 10 mammalian members of the Src family, Src Fyn and Yes are ubiquitously expressed, while Lck and Lyn have been detected in neurons as well as haemopoietic cells (Thomas and Brugge, 1997).

1.6.1. Src family signalling to the cytoskeleton.

The GTPase activating protein p190RhoGAP has preferential GAP activity for RhoA *in vitro* (Ridley et al., 1993) and is a major substrate for phosphorylation by c-Src both *in vitro* and *in vivo* (Brouns et al., 2001; Roof et al., 1998). In fibroblasts EGF receptor activation leads to Src kinase-dependent disassembly of stress fibres accompanied by redistribution of p190RhoGAP (Chang et al., 1995). Expression of the isolated GAP domain of p190RhoGAP in fibroblasts induces loss of constitutive stress fibres, and prevents RhoA-induced stress fibre assembly (Haskell et al., 2001; Ridley et al., 1993). The middle domain of p190RhoGAP appears to negatively regulate p190RhoGAP activity, and the major Src phosphorylation site in p190RhoGAP is situated in the middle domain (Haskell et al., 2001; Roof et al., 1998). Overexpression of Src prevents the negative effects of the middle domain, reconstituting the loss of stress fibres, suggesting that Src phosphorylation of p190RhoGAP at this site may increase p190RhoGAP activity* (Haskell et al., 2001). It should be noted however that Src-mediated phosphorylation of p190RhoGAP has no effect p190RhoGAP *in vitro*, and mutation of the Src phosphorylation site leads to only a minor reduction in GAP activity (Haskell et al., 2001).

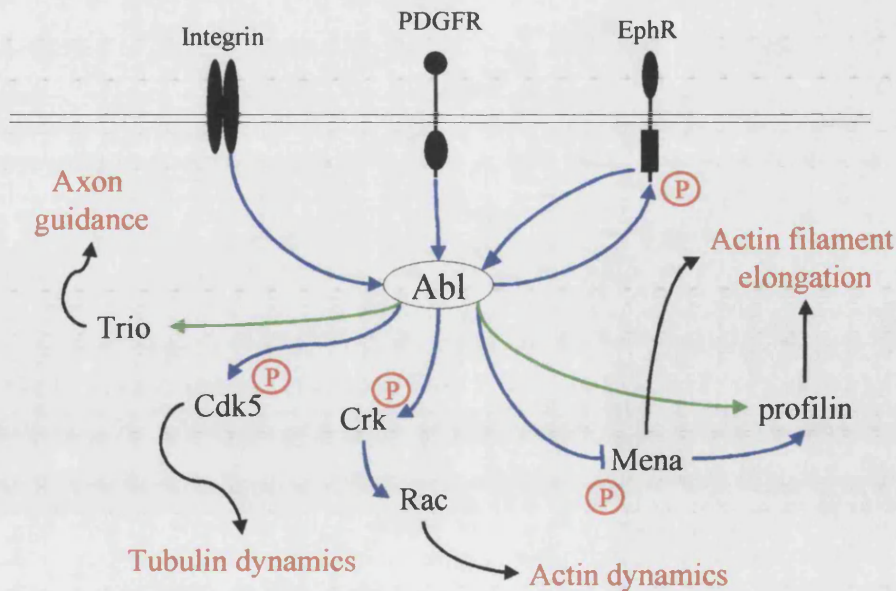
Despite these *in vitro* findings, there is a clear correlation between increased p190RhoGAP phosphorylation and decrease in RhoA activity (Arthur et al., 2000), and Src activity may negatively regulate p190RhoGAP phosphorylation via the low molecular weight protein tyrosine phosphatase (LMW-PTP)*. PDGF stimulation of fibroblasts leads to the Src-dependent phosphorylation of two LMW-PTP tyrosine residues that are known to positively regulate its phosphatase activity, and an associated reduction in the phosphorylation of p190RhoGAP (Bucciantini et al.,

Fig 1.10 Signalling by Src family kinases.



Src family kinases are regulated by the activation of various plasma membrane receptors, such as Integrins, EGF receptors and Eph receptors. Selected Src family substrates are shown (as indicated by **P**, to denote phosphorylation by kinases), together with the cellular responses mediated by these Src-dependent signalling pathways.

Fig 1.11. Signalling by Abl family kinases.



Abl family kinases are regulated by the activation of various plasma membrane receptors, such as Integrins, PDGF receptor and Eph receptors. Selected Abl family substrates are shown (as indicated by **P**, to denote phosphorylation by Abl kinases), together with the effect of this phosphorylation, and the cellular responses mediated by these Abl-dependent pathways. Biochemical interactions are shown in blue, genetic interactions in green.

1998; Chiarugi et al., 2000; Cirri et al., 1998; Rigacci et al., 1996). Together with the observation that a mutant of LMW-PTP that is not phosphorylatable by Src prevents PDGF-induced p190RhoGAP dephosphorylation, these data suggest that Src-stimulated LMW-PTP activity leads to dephosphorylation of p190RhoGAP (Chiarugi et al., 2000). It is not clear which site on p190RhoGAP is dephosphorylated by LMW-PTP, but Y1105 in its middle domain, the site of direct Src phosphorylation, appears to be the major phosphorylation site *in vivo* (Roof et al., 1998). Overall the experimental evidence suggests that Src activity has the potential to either increase or decrease phosphorylation of p190RhoGAP in response to receptor tyrosine kinase activation, and that this may activate or inhibit Rho activity to mediate changes in the actin cytoskeleton.

The regulation of actin dynamics by Src and p190RhoGAP is further complicated by the observation that Src-mediated phosphorylation of p190RhoGAP increases the association of p190RhoGAP with p120RasGAP (Roof et al., 1998). The association between these two enzymes has no effect on p190RhoGAP activity, but reduces p120RasGAP activity (Haskell et al., 2001; Roof et al., 1998). As mentioned in section 1.5.3 Ras activity antagonises Rho-ROCK-mediated stress fibre formation and prevents LPA-induced neurite retraction (Leblanc et al., 1998; Sahai et al., 2001). It is therefore possible that in addition to regulating p190RhoGAP activity, Src activation can modulate Rho-dependent rearrangements of the actin cytoskeleton, for example during axon guidance, via indirect changes in RasGAP activity (See Fig. 1.10).

1.6.2. Src family regulation of adhesion.

Src kinase activity plays a crucial role in regulating the turnover of cell-matrix adhesions during cellular migration by mediating degradation of proteins within the adhesion complex (Fincham and Frame, 1998; Frame et al., 2002; Webb et al., 2004). Fibroblasts deficient for Src, or all three ubiquitous Src family kinases, have much larger adhesion complexes than wild-type cells (Dumenil et al., 2000; Volberg et al., 2001). In addition Src-deficient fibroblasts exhibit stronger integrin-cytoskeletal links in response to vitronectin (Felsenfeld et al., 1999; Galbraith et al., 2002), suggesting that in wild-type cells Src acts to negatively regulate the strength of some cell-matrix adhesions.

The role of Src in regulating adhesion turnover has recently been directly addressed by examining the kinetics of the formation and disassembly of newly formed adhesions at the leading edge of migrating cells (Webb et al., 2004). Cells lacking Src Yes and Fyn assemble adhesions normally, but show a much reduced rate of adhesion disassembly (Webb et al., 2004). A series of experiments using a temperature-sensitive mutant of v-Src, the transforming product of Rous sarcoma virus that has constitutive kinase activity, has revealed a mechanism whereby Src activity regulates adhesion turnover by cleavage of FAK, which in turn results in the disassembly of the adhesion complex (Carragher et al., 2001; Carragher et al., 2003; Fincham et al., 1995). Inhibition of the calcium-dependent protease calpain prevents v-Src-induced FAK degradation and adhesion disassembly (Carragher et al., 2001; Carragher et al., 2002). A kinase-inactive mutant of v-Src does not lead to the degradation of FAK (Fincham and Frame, 1998), which may reflect the fact that v-Src activity increases calpain synthesis (Carragher et al., 2002). These observations provide strong evidence that v-Src activity induces the disassembly of adhesion complexes via increased calpain-mediated FAK proteolysis.

v-Src is similar to normal cellular Src in structure, except it lacks the regulatory c-terminal portion, but it is not clear that v-Src activity simply represents the behaviour of active cellular Src (c-Src). Fibroblasts deficient in c-Src, Yes and Fyn, or lacking c-Src kinase activity, do not disassemble cell-matrix adhesions normally (Webb et al., 2004), but to date there is no evidence that the c-Src kinases induce proteolysis of FAK. Interestingly in neurons Fyn activity enhances the proteolysis of NMDA receptor subunits by calpain (Rong et al., 2001), suggesting that in non-transformed cells, Src family kinases may have the potential to disassemble cell-matrix adhesions via calpain-mediated proteolysis.

1.6.3. Src family kinases in axon guidance.

Src and Fyn are highly expressed in the nervous system during development, and are enriched in developing axon tracts and neuronal growth cones *in vivo* (Bare et al., 1993; Bixby and Jhabvala, 1993; Cotton and Brugge, 1983; Fults et al., 1985; Maness et al., 1988). Given the ability of Src family kinases to regulate both cell-matrix adhesion and the actin cytoskeleton it seems likely that the kinase activity of these proteins may be involved in axon guidance.

Fyn has recently been shown to have a role in the cellular response to repulsive axon guidance cue Semaphorin3A (Sema3A). Sema3A is a diffusible axon guidance cue which acts to guide dorsal root ganglion (DRG) axons by repulsion, and induces collapse of DRG growth cones *in vitro* (Luo et al., 1995; Nakamura et al., 2000). Recently it has been reported that primary DRG neurons from Fyn homozygous null mice show reduced growth cone collapse in response to Sema3A *in vitro* (Sasaki et al., 2002). Both wild-type and constitutively active Fyn and c-Src lead to an increase in phosphorylation of the intracellular tail of the Semaphorin3A receptor Plexin-A2 (Sasaki et al., 2002), and v-Src expression has been shown to increase phosphorylation of the RCM, the mammalian homologue of UNC5 family of receptors known to mediate repulsive responses to the axon guidance cue Netrin (Tong et al., 2001). v-Src also and potentiates Netrin-induced RCM phosphorylation (Tong et al., 2001). Since phosphorylation is such a widespread mechanism both for regulating receptor function and for transducing intracellular signals, this data presents a model whereby Src family kinase activity may act to transduce or modulate repulsive axon guidance signals induced by activation of Semaphorin and Netrin receptors.

1.7. Abl family kinases.

Members of the Abl family of non receptor tyrosine kinases, including *Drosophila* Abl (D-abl), *C. elegans* and vertebrate c-Abl (cellular Abl) and vertebrate Arg (abl related gene) proteins, transduce information from growth factor, axon guidance and adhesion receptors to regulate changes in cell morphology and motility (Hernandez et al., 2004; Moresco and Koleske, 2003). Regulation of cytoskeletal dynamics is thought to underlie the role of Abl kinase activity in axon guidance (Bateman and Van Vactor, 2001; Lanier and Gertler, 2000) and therefore of the many potential Abl interacting proteins and/or substrates identified by genetic and biochemical techniques (Hernandez et al., 2004; Woodring et al., 2003) I will focus on those that have a role in regulation of the actin cytoskeleton and/or axon guidance.

1.7.1. Abl kinases and the actin cytoskeleton.

During development of *Drosophila* many dosage-sensitive modifiers of D-abl mutant phenotypes are known regulators of the actin cytoskeleton, for example the actin-

binding proteins chickadee/profilin and enabled (Ena), and the Rho-family GEF Trio (Gertler et al., 1990; Gertler et al., 1995; Liebl et al., 2000; Wills et al., 1999)*. Abl may act via these proteins to control cytoskeletal dynamics during development. For example during neuronal morphogenesis Abl/Arg null embryos exhibit buckling of the neural tube, which may result from the disordered F-actin bundles observed in these embryos (Koleske et al., 1998). Vertebrate Abl is also instrumental in mediating cytoskeletal rearrangement, for example following integrin engagement, which increases Abl kinase activity (Lewis et al., 1996; Woodring et al., 2001; Woodring et al., 2002). Increased Abl kinase activity correlates with formation of filopodia not only in adherent cells, but also in detached cells, suggesting that extracellular signals other than integrin engagement may mediate cytoskeletal rearrangements via Abl activity (Woodring et al., 2002). In agreement with this idea PDGF treatment of cells causes an increase in Abl kinase activity, and Abl-dependent membrane ruffling (Plattner et al., 1999). An elegant study by Ting and colleagues, using a fluorescent resonance energy transfer (FRET) indicates that active Abl localises to these PDGF-stimulated ruffles, suggesting a local regulation of actin structure by Abl kinase activity (Ting et al., 2001).

Abl family kinases may regulate actin rearrangement via direct actin binding, since the ability of Arg-GFP to direct the formation of F-actin structures in fibroblasts depend on Arg's F-actin binding domain (Wang et al., 2001). Both Abl and Arg can bundle actin filaments *in vitro*, and while Arg-dependent bundling requires only the presence of F-actin binding domains (Van Etten et al., 1994; Wang et al., 2001), the G-actin binding domain of Abl co-operates with the F-actin binding domain to bundle actin *in vitro* (Van Etten et al., 1994). Fragments of Arg lacking the kinase domain are still capable of bundling actin *in vitro* and inducing the formation of actin-rich structures *in vivo* (Wang et al., 2001), but Abl kinase activity is necessary for cytoskeletal rearrangement in other systems in response to RTK activation (Plattner et al., 1999), which suggests that Abl family kinases have additional roles in regulating cytoskeletal dynamics, other than actin bundling, likely involving interaction with and/or phosphorylation of substrates.

One major family of proteins known to link signalling pathways to actin dynamics is the Ena/VASP family, which consists of the product of the *Drosophila* enabled gene (Ena) and the vertebrate homologues Mena (Mammalian Enabled, VASP

(vasodilator stimulated phosphoprotein) and EVL (Ena-VASP like) (Gertler et al., 1996). Ena and Mena are substrates for D-Abl and Abl respectively *in vitro* and *in vitro* (Comer et al., 1998; Gertler et al., 1996; Tani et al., 2003) and genetic and biochemical evidence suggests that Abl kinases inhibit the function of Ena/VASP proteins by phosphorylation (Comer et al., 1998; Gertler et al., 1990; Gertler et al., 1995). Expression of the neural-specific isoform of Mena results in actin-rich membrane protrusions (Gertler et al., 1996), and VASP has an important role directing actin filament assembly during *Listeria* motility (Chakraborty et al., 1995) supporting an *in vivo* role for these proteins in actin dynamics.

Ena and VASP can directly bind G- and F-actin and promote actin filament nucleation *in vitro*, although the conditions under which this occurs are not physiological (Harbeck et al., 2000; Huttelmaier et al., 1999; Lambrechts et al., 2000). To date it is not clear how these properties contribute to Ena/VASP family function *in vivo*, since clustering of endogenous Ena/VASP proteins with peptide binding partners in living cells fails to nucleate or recruit actin filaments (Bear et al., 2000; Lasa et al., 1997; Pistor et al., 1994; Skoble et al., 2001). Instead sequestering Mena at the leading edge has a dramatic effect on the geometry and length of lamellipodial actin filaments (Bear et al., 2002). This is due to the ability of Mena to bind to uncapped actin filaments, preventing, or delaying their capping, and thus allowing actin filaments to increase in length (Bear et al., 2002).

Another potential link between Ena/VASP family proteins and the actin cytoskeleton is their ability to bind the actin binding protein profilin, both *in vitro* and in living cells (Gertler et al., 1996; Reinhard et al., 1995). Profilin promotes the formation of ATP-actin, and actin bound to profilin (profilactin) is preferentially added to the free barbed ends of actin filaments (Pollard and Borisy, 2003). Profilin binding sites of Ena/VASP proteins are situated within the central proline-rich domain (Ahern-Djamali et al., 1999; Gertler et al., 1996; Reinhard et al., 1995). The c-Abl phosphorylation site in Mena, and five out of six of the D-abl phosphorylation sites of Ena are also located in this proline-rich region (Comer et al., 1998; Tani et al., 2003). The functional consequences of Abl-mediated phosphorylation on Mena/profilin binding are unknown, but phosphorylation of Ena by D-abl results in disruption of Ena association with SH3-containing proteins that normally bind the proline-rich region (Comer et al., 1998). Interestingly mice heterozygous for both

profilin and Mena show a far more severe phenotype than loss of Mena alone, suggesting that Mena/profilin interactions might also be important in vertebrates (Lanier et al., 1999). In addition mutations in the *Drosophila* profilin gene chickadee give rise to a very similar phenotype to that seen in D-abl^{-/-} mutants, and D-abl and chickadee show dosage sensitive genetic interactions (Wills et al., 1999). Taken together these observations suggest that Abl-mediated phosphorylation of Ena/VASP family proteins may influence the actin cytoskeleton via profilin, and that these interactions are likely to be important *in vivo*.

Abl and Arg have also been shown to phosphorylate the SH2/SH3 domain containing adaptor protein Crk, and this phosphorylation event leads to an intramolecular interaction between the phosphotyrosine residue and the SH2 domain, preventing Crk interaction with some of its effectors (Escalante et al., 2000; Feller et al., 1994; Wang et al., 1996). Abl-dependent Crk phosphorylation correlates with a decrease in Crk association with CAS, whereas loss or inhibition of Abl kinase activity increases Crk-CAS coupling (Kain and Klemke, 2001). Crk binds the Rac GEF DOCK180 via its SH3 domain (Matsuda et al., 1996), and co-expression of Crk and CAS promotes DOCK180-mediated increase in Rac activity and associated actin rearrangements (Kiyokawa et al., 1998). Although the effect of uncoupling Crk-CAS on DOCK180 and Rac activity was not investigated in this study, it is possible that Abl phosphorylation of Crk, and the resulting uncoupling of the Crk-CAS complex, might antagonise DOCK180-mediated Rac activation and subsequently have modulatory effects on the actin cytoskeleton (See Fig. 1.11).

1.7.2. Abl kinases in axon guidance.

Expression of constitutively active Abl in neurons in culture leads to the formation of filopodia along the length of the axon, and increases axon outgrowth, providing evidence that Abl activity can modulate the neuronal cytoskeleton (Woodring et al., 2002; Zukerberg et al., 2000). Abl and Arg are expressed in many regions of the mammalian brain, and in the growth cones of primary mammalian neurons *in vitro*, but genetic manipulations have failed to reveal any axon guidance defects in the absence of mammalian Abl and Arg. Viable double heterozygous knockout animals show no gross neuronal defects, and double homozygous knockouts die from defects in neural tube closure (Koleske et al., 1998).

Much of the evidence that implicates Abl kinases in axon guidance comes from genetic studies in *Drosophila*. Axons in the embryonic CNS of *Drosophila* are organised into two major groups; longitudinal pathways that extend along the anterior-posterior axis, and commissural axons that project across the midline. The midline glia secrete the chemorepellent Slit, and the Slit receptor Robo is highly expressed on the longitudinal axons, preventing them from crossing the midline, and the commissural axons only after they have crossed the midline, preventing them from re-crossing (Guthrie, 2001; Van Vactor and Flanagan, 1999). D-abl homozygous loss-of-function mutants exhibit aberrant midline crossing of longitudinal axons in the developing *Drosophila* CNS (Hsouna et al., 2003; Wills et al., 1999; Wills et al., 2002). The CNS scaffold in these animals resembles that observed in heterozygous Slit or Robo mutants (Hsouna et al., 2003) suggesting that D-abl co-operates with a repulsive axon guidance cue at the midline. Consistent with this idea, heterozygous mutations in Robo and Slit enhance the Abl mutant phenotype, and the low frequency of aberrant axon crossings in animals heterozygous for Slit is increased 20 fold with an additional D-abl loss-of-function mutation (Hsouna et al., 2003; Wills et al., 2002). Robo heterozygous loss of function mutations show a higher frequency of midline errors than slit, especially if multiple Robo receptors are reduced in dose, but these loss of function phenotypes are also enhanced by Abl loss of function (Wills et al., 2002). Overexpression of wild-type or a hyperactive mutant of Robo can suppress the aberrant axon crossings seen in Abl loss of function mutants (Hsouna et al., 2003). Since a 'kinase dead' mutant of D-abl cannot rescue the D-abl phenotype it seems likely that Abl kinase activity is necessary for the co-operative role of D-abl in mediating repulsive axon guidance at the midline following Slit-Robo interaction (Wills et al., 2002).

Although to date there is no evidence that Abl kinase is involved in axon guidance in vertebrates, Slit appears to function in guidance of callosal axons at the mouse midline, a process which requires the Abl substrate Mena (Bagri et al., 2002; Lanier et al., 1999), and this suggests that the role of Abl in mediating repulsive axon guidance signals may be conserved in mammals.

*(in which the lysine residue within the ATP-binding domain of the Abl kinase domain has been replaced with asparagine, eliminating Abl tyrosine kinase activity)

1.7.2.1. Ena/VASP family proteins in axon guidance.

Ena was first identified as a suppressor of the CNS defects in D-abl mutants (Gertler et al., 1990). Mutations in Ena have also been shown to suppress the motor neuron bypass phenotype and increased longitudinal axon crossing seen in D-Abl loss of function mutants (Wills et al., 1999; Wills et al., 2002), suggesting that Ena function antagonises Abl activity during axon guidance in *Drosophila*. There is also evidence that vertebrate Ena/VASP proteins play a role in axon guidance. Mice deficient for Mena have a defective corpus callosum, since cortical axons, having projected normally to the midline, fail to cross (Lanier et al., 1999). Although genetic studies are clearly useful for identifying potential signalling mediators, the results do not give rise to a unifying model for the role of Abl and Ena/VASP families in axon guidance, since genetic studies have also implicated Ena in mediating attractive guidance cues (Gitai et al., 2003; Yu et al., 2002). Biochemical interactions and *in vitro* assays might help to elucidate the role of Ena/VASP proteins and Abl kinase during axon repulsion, for example mediated by Eph receptors, which have been shown to interact with Abl (Yu et al., 2001).

1.7.2.2. Cdk5 and axon guidance.

The cyclin-dependent kinase Cdk5 is expressed in post mitotic neurons, and plays a major role in the positioning of certain neuronal sub-populations during cortical development (Ohshima and Mikoshiba, 2002). Recent evidence has implicated Cdk5 in the repulsive neuronal response to both Sema3A and ephrin-A5 (Cheng et al., 2003; Sasaki et al., 2002). Cdk5 is phosphorylated by c-Abl *in vitro*, and expression of activated c-Abl results in increased cellular levels of phosphorylated Cdk5 (Zukerberg et al., 2000). It is likely that the adaptor cables is important for Abl-induced Cdk5 activity, c-Abl and Cdk5 do not associate in the absence of cables, and the presence of cables potentiates Abl-mediated phosphorylation of Cdk5 (Zukerberg et al., 2000).

Abl, cables and Cdk5 form a trimolecular complex *in vitro*, and colocalise in the growth cone of neurons grown in culture (Zukerberg et al., 2000), which is the region in which dynamic actin rearrangements occur in during the response to ephrin-A stimulation (Meima et al., 1997a; Meima et al., 1997b). Importantly phosphorylation by Abl increases the kinase activity of Cdk5 (Sasaki et al., 2002; Zukerberg et al.,

2000), and recent data suggests that Cdk5 activity may mediate the repulsive response of RGCs to ephrin-A5, since pharmacological inhibition of Cdk5 in temporal RGCs inhibits ephrin-A5-induced growth cone collapse (Cheng et al., 2003). Although no functional interaction between Eph receptors and Cdk5 has been demonstrated to date, it is possible that Eph receptor activation may lead to increased Cdk5 activity in these neurons, and that this contributes to ephrin-induced growth cone collapse. Given that Abl kinase phosphorylates and thus activates Cdk5, and that Eph receptors have been shown to interact with Abl (Yu et al., 2001; Zukerberg et al., 2000), the above observations raise the intriguing possibility that EphA receptors may mediate repulsive axonal responses via Abl kinase activity.

1.8. Aims of this thesis.

The main aim of this thesis is to identify signalling molecules that mediate ephrin-A-induced repulsion of RGCs. Although ephrin-As are membrane bound molecules *in vivo* (Gale et al., 1996b; Pandey et al., 1995b), previous studies have used soluble ephrins to identify signalling molecules that play a role in Eph receptor mediated signals. I have therefore developed a physiologically relevant co-culture assay, in which the response of an individual RGC growth cone to contact with an ephrin-A-expressing cell is followed dynamically. Previously, the Rho GEF Ephexin, Rho and the Rho effector ROCK have been implicated in the response of RGCs to soluble ephrin-A (Cheng et al., 2003; Shamah et al., 2001; Wahl et al., 2000). I have used the co-culture assay to investigate the role of ROCK in the cellular response of RGCs to contact with an ephrin-A-expressing cell.

Inhibition of Rho, ROCK or Ephexin results only in partial inhibition of the RGC response to soluble ephrin-A (Cheng et al., 2003; Shamah et al., 2001; Wahl et al., 2000), suggesting that other signalling intermediates play a role in mediating neuronal EphA-dependent signals. In order to test the hypothesis that non-receptor tyrosine kinases of the Src and Abl family are involved in neuronal EphA-mediated signalling pathways, I have investigated the effect of well-characterised pharmacological inhibitors of these kinases on the dynamic RGC response to stimulation with ephrin-A, and present evidence that Abl family kinases mediate all aspects of the RGC response to stimulation with ephrin-A.

Chapter 2. Materials and Methods.

2.1. Media and solutions.

Unless otherwise stated all general laboratory chemicals are from Sigma.

2.1.1. Cell media.

RGC culture medium.

8% v/v Foetal bovine serum	Gibco
2% v/v Chicken serum	Sigma
1% v/v Penicillin/streptomycin	Gibco
2mM L-Glutamine	Gibco
in DMEM/F12-HAM	Sigma

DRG culture medium.

10% Foetal bovine serum	
0.2% w/v Na Bicarbonate	
0.7% w/v Glucose	
2mM L-Glutamine	
1% v/v Penicillin/streptomycin	
in L-15 medium	Gibco

Cell line medium.

10% v/v Foetal bovine serum	
1% v/v Penicillin/streptomycin	
in DMEM	

2.1.2 Cell fixation and coverslip mountant.

Cytoskeletal buffer.

10mM MES pH6.1

125mM KCl

3mM MgCl

2mM EGTA

10% w/v Sucrose

Mowial mountant.

25% v/v glycerol

0.1M Tris-HCL pH8.5-8.8

10% w/v Mowial

Calbiochem

2.1.3 Solutions for biochemistry

TBS.

20mM Tris-HCL pH7.4

150mM NaCl

RIPA buffer.

20mM Tris-HCL pH7.4

150mM NaCl

1% v/v Triton X-100

0.5% w/v Na deoxycholate

0.1% w/v SDS

10% v/v Glycerol

5mM EDTA

50mM NaF

0.5mM NaVO₃

Complete Protease Inhibitor tablet (Roche)

100 μ gml⁻¹ PMSF

IP buffer.

20mM Tris-HCL pH7.4

1% v/v NP-40

10% v/v Glycerol

50mM NaF

0.5mM NaVo₃

Complete Protease Inhibitor tablet

100 μ gml⁻¹ PMSF

Running buffer.

25mM Tris-HCL

192mM Glycine

0.1% w/v SDS

Transfer buffer.

48mM Tris-HCL

39mM Glycine

0.037% w/v SDS

20%v/v Methanol

2.2. Cell culture.

2.2.1. Coating of coverslips and tissue culture dishes.

13- or 22- mm diameter glass coverslips (BDH) were washed in concentrated Nitric acid in a 1L conical flask for 5 min, washed under running tap water for 10 min and then rinsed twice in distilled water. After a final wash in methanol the coverslips were transferred to a glass Petri dish and baked at 120°C for 4 hours. Coverslips were transferred under sterile conditions to a 9 cm plastic Petri dish containing 25 ml of

1mgml⁻¹ poly-L-lysine (pLL; Fluka). The dishes were sealed with Parafilm and the coverslips were incubated overnight at room temperature on an orbital shaker, the pLL was removed (and retained for re-use at 4°C for a maximum of 3 times) and the coverslips rinsed three times with distilled water. The coverslips were washed overnight with distilled water on a orbital shaker for 3 consecutive nights, with the water changed each morning. The coverslips were then dried overnight by balancing them on edge around the edge of a dry 9 cm plastic Petri dish in a sterile tissue culture hood. Dry laminin-coated coverslips were stored in clean 9 cm plastic Petri dishes, sealed with Parafilm for up to 4 weeks. Prior to use, pLL-coated coverslips were placed in a dry clean 9 cm plastic Petri dish and 500µl of 100µgml⁻¹ laminin (Sigma) in DMEM (Gibco) was added to each 22 mm coverslip (200µl to each 13 mm coverslip). A second pLL-coated coverslip was placed on top of each coverslip, sandwiching the laminin solution between the coverslip sandwiches were incubated overnight at 37°C and 5% CO₂ in a humidified tissue culture incubator, then each coverslip was rinsed in sterile DMEM and placed, laminin-side up, into one well of a six-well (22 mm coverslips) or 4-well (13 mm coverslips) tissue culture dish (both Nunc). Approx. 100µl of the appropriate growth medium was added to each coverslip while cells were prepared, to avoid the coverslips drying out.

For isolated RGCs (see section 2.1.3) 9 cm tissue culture dishes (Nunc) were incubated with 25 ml of 1mgml⁻¹ pLL solution overnight on an orbital shaker. Dishes were rinsed three times in sterile phosphate-buffered saline (PBS) and used immediately for laminin coating (see below) or left to dry overnight in a sterile tissue culture hood before being stored in sealed bags containing desiccating agent (Sigma). 6 ml of 100µgml⁻¹ laminin-DMEM was placed in each pLL-coated tissue culture dish, the dishes sealed with Parafilm and incubated at room temperature overnight on an orbital shaker. The laminin solution was removed and retained at 4°C for re-use (maximum three times) and the dishes were washed three times with sterile DMEM and then incubated with 10 ml RGC medium at 37°C and 5% CO₂ in a humidified tissue culture incubator until the isolated RGCs (see section 2.1.3) were ready for plating.

2.2.2. Retinal explants.

Fertile eggs were obtained from Henry Stewart and Co. (Fakenham, UK). Eggs were rinsed with 70% ethanol and E7 embryos were placed in a 9 cm tissue culture dish (Nunc) in RGC medium and sacrificed by decapitation using size 2 forceps. The eyeballs were removed by gently pinching them out with forceps (size 2) and placed in RGC medium on ice while not in use. Each eyeball was placed on a dry dish for removal of the mesenchyme and the pigment layer under a dissecting microscope (Zeiss), using size 5 forceps at a shallow angle to avoid tearing the retina. The eyeball was then transferred to a 9 cm dish containing RGC medium for removal of the vitreous body by inserting size 5 forceps into the lens hole and drawing out the vitreous humor between a second pair of open size 5 forceps. The nasal third of the retina was separated from the rest, which was discarded. A strip of black mixed-cellulose filter (HABP filter, Millipore) was placed in the dish and slid under the sheet of retina. The retina was gently unrolled with closed forceps and attached, RGC layer uppermost, to the filter by pressing the edge of the retina down with closed forceps. The flat-mounted retina was cut into 400 nm-wide strips using a McIlwain tissue chopper (Mickle Laboratory Engineering Company Ltd). 20 μ L of RGC medium was spread over the surface of the removable cutting disk and the flat-mounted retina was placed onto the disk with the nasal-temporal axis parallel to the blade. Approx. 50 μ L of RGC medium was added to the flat-mounted retina after the strips were cut to avoid the tissue drying out. Individual retinal strips were transferred to a six-well tissue culture dishes (Nunc) or 35 mm glass-bottomed dishes (World Precision Instruments), containing a 22 mm pLL/laminin coated coverslip in each well, using size 5 forceps to grip the end of the cellulose strip where there is no retinal tissue. The retinal strips were placed RGC layer down onto the coverslips. Typically two strips were placed on each 22 mm diameter coverslip, and anchored with small stainless-steel bars (approx. 10 x 1 x 1 mm in dimension). 2 ml of RGC medium was added to each well. The retinal explants were incubated for 20-26 hours in RGC medium at 37°C and 5% CO₂ in a humidified tissue culture incubator.

2.2.3. Isolated RGC cultures.

This method of isolating RGCs is based on a protocol described by de Curtis (1991) and depends on the difference in density of these cells compared to other retinal

cells. 10-20 E7 chick retinae were dissected as described in section 2.1.2, but instead of being flat-mounted on cellulose filters the retinae were incubated on ice in a 15 ml centrifuge tube (Falcon) in RGC medium. RGC medium was removed by aspiration and Retinae were washed twice with sterile PBS. Retinae were allowed to settle by gravity between and after washes. PBS was removed by aspiration and the retinae were incubated with 2.2% hyaluronidase (Sigma) in PBS (1 ml total volume) in a water bath for 5 min at 37°C. Retinae were spun in a swinging rotor centrifuge at 500g for 3 min, washed with sterile PBS and spun again at 500g for 3 min. All supernatant was removed and the retinae were then incubated in 0.1% trypsin and 0.01% DNAase I type IV (both Sigma) in sterile PBS (total volume 5 ml) in a water bath for 8 min at 37°C, during which time the tube was inverted every 2 min. 5 ml of RGC medium was added to inhibit the trypsin, together with a further 0.01% DNAase I type IV, and the cells were spun at 500g for 3 min. Supernatant was removed and the cells were gently resuspended in 5 ml RGC medium, spun again and resuspended in 0.2% soybean trypsin inhibitor (Sigma) and 0.05% DNAase I type IV in RGC medium (2 ml total volume) at room temperature. The cells were triturated slowly (8 aspirations and expulsions lasting about 2 seconds each) using a fire polished pipette with a 1 ml bulb, and the cells were diluted with RGC medium to a total volume of 12 ml.

The Percoll (Amersham) gradient was prepared by mixing layers of decreasing density (Table 2.1) at room temperature in a 15 ml centrifuge tube (Falcon), and slowly pipetting each layer in the following order into a 50 ml centrifuge tube (Falcon).

The gradient was spun in a cooled, swinging rotor centrifuge for 30 min at 500g and 5°C with minimum acceleration and deceleration. The higher of the two bands of isolated cells, which is enriched in RGCs (de Curtis et al., 1991), was collected with a fire polished pipette. RGC medium was added to a final volume of 10 ml and the cells were spun at 500g for 8 min, before being resuspended in 1 ml RGC medium and pre-plated on a 9 cm tissue culture dish at 37°C for 3-5 hours to remove any remaining non-neuronal cells. RGCs were removed from the dish by pipetting and spun at 500g for 3 min. Approx. 10^6 RGCs were plated onto a 9 cm laminin-coated tissue culture dish (see section 2.1.1). Isolated RGCs were grown in RGC medium at 37°C and 5% CO₂ for 20-26 hours in a humidified tissue culture incubator.

Table 2.1. Preparation of Percoll gradient.

Layer	DMEM-F12 HAM	Cell suspension	Percoll
5	4 ml	0 ml	5.65 ml
4	2 ml	4 ml	3.8 ml
3	4 ml	4 ml	1.75 ml
2	4 ml	4 ml	0.842 ml
1	4 ml	0 ml	0 ml

2.2.4. DRG explants.

Fertile eggs were rinsed with 70% ethanol, and the E7 embryos placed in L-15 medium (Gibco). E7 embryos were sacrificed by decapitation using size 2 forceps and eviscerated using size 5 forceps at a shallow angle to avoid damaging the tissue either side of the spinal cord. The limbs and tail were pinched off using size 5 forceps and the torso transferred with forceps to a silicon-coated dish containing L-15 medium. The torso was placed dorsal side up and, viewed under a dissecting microscope, was pinned flat by inserting dissecting pins either side of the spinal cord. The torso was split longitudinally along the centre of the spinal cord with a microsurgical knife (World Precision Instruments) each half was unpinned and reoriented with the newly cut surface uppermost. After pinning the half-torso in position the spinal tissue and myelin were peeled away from each cut surface of the embryo using size 5 forceps, and the ganglia removed from the region between the limbs by pinching the nerve root between size 5 forceps. Each DRG was divided into two using size 5 forceps and placed onto the centre of a 13 mm pLL/laminin coated coverslip in a 4-well tissue culture dish (Nunc) in 600 μ l of DRG medium. Most of the medium was removed with a pipette to bring the DRG explant into contact with the coverslip and allow it to attach to the laminin. After approx. 30 min incubation at room temperature in minimal volume, DRG medium was gently added to each well to a final volume of 600 μ l, and the explants incubated for 20-26 hours at 37°C and 5% CO₂.

2.2.5. Cell lines.

Swiss-3T3 fibroblasts.

Swiss 3T3 fibroblast cells were grown in cell line medium in 9 cm tissue culture dishes (Nunc). At 60-80% cell confluency the medium was removed by aspiration and the cells were washed twice with trypsin-EDTA (Gibco) and incubated with minimum volume of trypsin-EDTA in a humidified tissue culture incubator for 2 min at 37°C. 4 ml cell line medium was added to inhibit the trypsin and dislodge the cells, which were then transferred to a 15 ml centrifuge tube (Falcon) and spun in a swinging rotor centrifuge at 2000 rpm for 1 min. The medium was removed and the cells resuspended in 1 ml cell line medium. The cells were re-plated at dilutions between 1:4 and 1:10, in fresh cell line medium in a clean 9 cm tissue culture dish, or approximately 4000 Swiss-3T3 fibroblasts were plated on a 22 mm pLL/laminin coated coverslip with or without retinal explants, for use in the co-culture assay or for immunofluorescence studies respectively (see below). Swiss 3T3 fibroblasts were cultured in a humidified tissue culture incubator at 37°C and 5% CO₂.

MDCK cells.

MDCK cells were grown in cell line medium in 9 cm tissue culture-dishes (Nunc). At 100% confluency the medium was removed by aspiration and the cells were washed twice with trypsin-EDTA (Gibco) and incubated with 2 ml trypsin-EDTA at room temperature for 10 min to disrupt cell-cell junctions, before being transferred to a humidified tissue culture incubator for 5 min at 37°C. 4 ml cell line medium was added to inhibit the trypsin and dislodge the cells, which were then transferred to a 15 ml centrifuge tube (Falcon) and spun in a swinging rotor centrifuge at 2000 rpm for 1 min. The MDCK cells were resuspended and re-plated on 9 cm tissue culture dishes or pLL/laminin-coated coverslips as described above for Swiss-3T3 fibroblasts. MDCK cells were cultured in a humidified tissue culture incubator at 37°C and 5% CO₂.

COS cells.

COS cells were grown in cell line medium in 9 cm tissue culture-dishes (Nunc). At 80-90% confluency the medium was removed by aspiration and the cells were

washed twice with trypsin-EDTA (Gibco) and incubated with 2 ml trypsin-EDTA in a humidified tissue culture incubator for 2 min at 37°C. 4 ml cell line medium was added to inhibit the trypsin and dislodge the cells, which were then transferred to a 15 ml centrifuge tube (Falcon) and spun in a swinging rotor centrifuge at 2000 rpm for 1 min. The COS cells were resuspended in 1 ml of cell line medium and plated on 9 cm tissue culture dishes at dilutions between 1:2 and 1:10, or into 6 well plates (Nunc) for transfection experiments (see below). COS cells were cultured in a humidified tissue culture incubator at 37°C and 5% CO₂.

2.3. Microinjection and transfection techniques.

2.3.1. Expression plasmids

prk5-EphA4 was a gift from David Wilkinson (NIMR, London) and pCDM8-ephrin-A5 was a gift from Uwe Drescher (King's College, London).

2.3.2 Microinjection.

Retinal-MDCK co-cultures were placed in RGC medium on the heated stage of an inverted microscope (Zeiss) and viewed with 5x and 32x phase contrast air objectives. The cells were maintained at 37°C and 5% CO₂ throughout. A solution of 0.06 mgml⁻¹ DNA and 0.1 mgml⁻¹ FITC-dextran (as an injection marker) in filtered PBS was loaded with microloader pipette tips (Eppendorf) into boro-silicate needles, pulled with a horizontal needle puller (Suter). The needle was inserted into the nucleus of MDCK cells surrounding the retinal explant using a micromanipulator (Eppendorf) and DNA was injected with a Transjector pump (Eppendorf). Co-cultures were replaced in a humidified tissue culture incubator at 37°C and 5% CO₂, for 3-4 hours to allow for expression. Injected MDCK cells in the proximity of growing RGC axons were identified by briefly scanning the co-culture for FITC-dextran-positive cells under a 5x objective, and the resulting interactions were analysed by phase time-lapse microscopy (see section 2.5.1).

2.3.3. Transfection of COS cells.

COS cells, at 50-60% confluency, growing in cell line medium in 6-well plates (Nunc) were transfected using GeneJuice (Merk Biosciences). For each well 3µl of GeneJuice was mixed with 100µl DMEM and incubated at room temperature for 5

min before 1µg of DNA was added to the cells and the mixture incubated for a further 10 min. The DNA-GeneJuice complex was added drop-by-drop and mixed by gentle rocking. Cells were cultured for 24 hours in a humidified tissue culture incubator at 37°C and 5% CO₂ prior to use in experiments.

2.4. Anti-phospho-Eph receptor antibody.

Anti-phospho-Eph receptor antibodies were raised in rabbit against the peptide sequence LRTYpVDPHTYpEDPTQ. This sequence is taken from the juxtamembrane region of EphA3, in which both of the juxtamembrane tyrosine residues, which are highly conserved throughout the Eph receptor family, are phosphorylated (Fox et al., 1995; Sajjadi and Pasquale, 1993; Shamah et al., 2001). Phosphorylation of these two tyrosine residues, which are major autophosphorylation sites *in vitro* and *in vivo*, is required for full Eph kinase activity (Binns et al., 2000; Choi and Park, 1999; Ellis et al., 1996; Kalo et al., 2001; Kalo and Pasquale, 1999; Wybenga-Groot et al., 2001; Zisch et al., 1998). In addition to recognising phosphorylated EphA3 (Shamah et al., 2001) this antibody recognises phosphorylated EphB2, EphB4, EphA4 and EphA7 (Marston et al., 2003; DJ Marston and CD Nobes, unpublished data). Unpublished work from the lab has also shown that this antibody does not recognise unphosphorylated Eph receptors, and that antibody binding can be inhibited by pre-incubation with the phospho-peptide antigen (DJ Marston, unpublished data).

It should be noted that the anti-phospho-specific Eph receptor antibody has not been affinity purified against the peptide substrate, and it is therefore formally possible that this antibody recognises phosphorylated tyrosine residues on proteins other than Eph receptors, or indeed phosphotyrosine residues within the Eph receptor sequence other than the juxtamembrane tyrosines. If this were the case, the staining pattern revealed when using this antibody for immunocytochemistry or Western blotting (see sections 2.5 and 2.9) might reflect tyrosine phosphorylation of other signalling molecules within RGCs or COS cells, in addition to phosphorylation of Eph receptors on the residues that correlate with increased Eph kinase activity.

2.5. Immunocytochemistry.

Cell medium was removed by aspiration and cells were fixed with 4% formaldehyde in cytoskeletal buffer (Symons and Mitchison, 1991) with or without 0.2% glutaraldehyde for 10 min at room temperature. Coverslips were rinsed 3 times in PBS. In the case of retinal explants the fixed axons were cut close to the explant tissue using a microsurgical knife after fixation, and the explant removed to minimise autofluorescence. Coverslips were incubated with 0.2% triton-X 100 for 5 min and then rinsed 3 times in PBS. 0.5 mgml^{-1} Na borohydride was added for 10 min and cells were rinsed 3 times with PBS. The primary antibody was added to the coverslip for 45-60 min at room temperature. Coverslips were rinsed 3 times in PBS and then washed over 15-30 min with a minimum of 3 changes of PBS. Secondary antibodies were added to the coverslip for 45 min at room temperature. F-actin was visualised with rhodamine-labelled phalloidin, included with the secondary antibody. Coverslips were incubated with secondary antibody and/or phalloidin for 45 min, the coverslips were rinsed 3 times in PBS and then washed over 30-60 min with a minimum of 3 changes of PBS. Coverslips were rinsed in distilled water and mounted on Microscope slides with Mowial mountant containing phenylenediamine as an anti-fade agent. All dilutions were made in PBS.

In order to visualise ephrin-As on the surface of Swiss-3T3 fibroblasts and MDCK cell in culture, EphA5 (R & D Systems) at a concentration of $4 \mu\text{gml}^{-1}$ in RGC medium was incubated with a 5-fold excess of goat anti-human IgG (Stratech) for 20 min in a humidified tissue culture incubator at 37°C and 5% CO_2 . Clustered EphA5-Fc was added to the bathing medium at a final concentration of $1 \mu\text{gml}^{-1}$ for 10 min prior to fixation as described above, and the EphA5-Fc visualised using FITC-labelled anti-goat antibody.

In order to visualise endogenous, phosphorylated Eph receptors present on RGCs in culture, cells were fixed, permeabilised and quenched as described above. Anti-phospho-Eph receptor antibody (at a dilution of 1:300) was detected using a tyramide amplification system (Molecular Probes).

Table 5.2. Commercially available antibodies used for immunocytochemistry.

Antibody	Species	Dilution	Source
Ephrin-A5	Goat	1:250	R & D Systems
FITC anti-Goat	Donkey	1:300	Jackson Immuno-Laboratories
FITC anti-Rabbit	Donkey	1:300	Jackson Immuno-Laboratories
Rhodamine anti-Goat	Donkey	1:300	Strattech
Rhodamine Phalloidin [†]		1:250 (neurons) 1:500 (cell lines)	Sigma

[†]Rhodamine Phalloidin was diluted to a stock concentration of 50 μ gml⁻¹ in Methanol and stored at -20°C.

2.6. Cell imaging.

2.6.1 Phase time-lapse microscopy.

Retinal explants and co-cultures were placed in RGC medium in a 35 mm glass-bottomed dish (World Precision Instruments) on the heated stage of an inverted microscope (Zeiss) and viewed using 5x and 32x phase contrast air objectives. The cells were maintained at 37°C and 5% CO₂ throughout. Time-lapse images were collected using the 32x objective with the addition of a 1.6x optivar lens, using an Orca ER camera (Hamamatsu) and Openlab software (Improvision). Movies of soluble ephrin-A5-Fc-induced RGC growth cone collapse were collected one frame every 10 seconds, with the exception of movie 4.5 (in the presence of Y27632) which was filmed at one frame every 12 seconds. RGC-fibroblast/MDCK interactions were filmed at one frame every 15 seconds with the exception of movies 4.6 and 4.9 (RGC-fibroblast interactions in the presence of PP2 and PP3 respectively) which were filmed at 1 frame every 20 seconds. During the period of recording poly(dimethylsiloxane) 200 mineral oil (Aldrich) was placed on top of the

medium to prevent evaporation. After recording the oil was removed with a pipette from the RGC-MDCK co-cultures that would be fixed and stained to check for ephrin-A5 expression.

2.6.2. Confocal microscopy.

Fluorescent images were obtained using an upright confocal microscope (Leica) using a 63x oil immersion objective. The images were processed using the Leica confocal software and PhotoShop (Adobe).

2.7. Cell assays.

2.7.1. Co-culture assay.

Swiss-3T3 fibroblasts or MDCK cells were trypsinised as described in section 2.1.3 and added to coverslips on which retinal explants had previously been cultured for 18-24 hours (Swiss-3T3 fibroblasts) or 10-16 hours (MDCK cells). Co-cultures were incubated for 2-4 hours (Swiss 3T3 fibroblasts) or 8-10 hours (MDCK cells) at 37°C and 5% CO₂ in a humidified tissue culture incubator before being transferred to the heated stage of an inverted microscope (Zeiss). Co-cultures were viewed under a 32x objective to identify potential RGC-Fibroblast/MDCK cell interactions. Time-lapse phase microscopy recordings of cell-cell interactions were made as described in section 2.5.1. RGC responses to cell contact were quantified under two sets of strict criteria. Loss of lamella is defined as the complete loss of RGC growth cone lamella for more than 1 min. Axon retraction is defined as the phase-dark region in the central domain of the RGC growth cone, which is revealed as the axon terminal when the RGC lamella is lost, retracting beyond its position at the point of initial cell contact. Initial contact is defined as contact between the lamella of the RGC growth cone and the lamella of the contacting cell. Withdrawal of the contacting cell is defined as the non-neuronal cell retracting more than 10µm from the point of contact with a RGC growth cone. Non-neuronal cells that were migrating prior to the interaction were not quantified for withdrawal. Interactions were recorded for a minimum of 25 min after initial contact, unless axon retraction had occurred before this time.

To detect RGC Eph receptor phosphorylation induced by contact with a Swiss-3T3 fibroblast, fibroblasts were added to retinal explants as described above and after 2-4 hours the co-cultures were fixed and stained with fluorescent phalloidin and anti-phospho-Eph receptor, as described in section 2.4. Images of RGCs alone or in contact with a Swiss-3T3 fibroblast were captured by confocal microscopy (see section 2.5.2).

2.7.2. Soluble growth cone collapse assay.

Retinal or DRG explants were cultured as described in section 2.1. Soluble ephrin-A5-Fc (R & D Systems) was clustered into higher order oligomers by incubation of ephrin-A5-Fc ($4\mu\text{gml}^{-1}$) in RGC medium with a 5-fold excess of goat or rabbit anti-human IgG (Strattech) for 20 min in a humidified tissue culture incubator at 37°C and 5% CO_2 . Anti-human IgG was incubated in the same way, but in the absence of ephrin-A5-Fc as a control. Clustered ephrin-A5-Fc at a final concentration of $1\mu\text{gml}^{-1}$, or anti-IgG at a final concentration of $5\mu\text{gml}^{-1}$, was added to the bathing medium of retinal explants, gently mixed by pipetting and the retinal explants were incubated with ephrin-A5-Fc at 37°C and 5% CO_2 for 10 min in a humidified tissue culture incubator. Sema3A (R & D Systems) was added to the bathing medium of DRG explants at a final concentration of 1 or $5\mu\text{gml}^{-1}$ and the DRG explants incubated at 37°C and 5% CO_2 for 10 min in a humidified tissue culture incubator. The explants were then fixed, stained with Rhodamine-labelled phalloidin and mounted on microscope slides as described above. RGC and DRG axons were viewed under an inverted fluorescence microscope (Axioscope) using a 63x oil immersion objective and were quantified under three strict sets of criteria with regards the morphology of their F-actin cytoskeleton. Full growth cone defines axons that have visible growth cone lamella. Loss of lamella defines axons that exhibit no visible growth cone lamella and have more than three F-actin positive filopodia or retraction fibres. Total collapse defines axons that have 3 or fewer filopodia/retraction fibres. More than 100 axons were counted in each experiment, and at least three repeat experiments were performed.

RGC responses to soluble $1\mu\text{gml}^{-1}$ clustered ephrin-A5-Fc recorded by phase time-lapse microscopy were quantified for loss of lamella and axon retraction as in the co-

culture assay, but times were measured from the addition of ephrin-A5-Fc to the bathing medium.

2.8. Pharmacological inhibitors.

Pharmacological inhibitors, or the equivalent volume of the appropriate carrier, were added to the bathing medium of retinal or DRG cultures at the concentrations and stated below. Y27632 was added 1 hour prior to the addition of soluble ephrin-A5-Fc, and 1-2 hours prior to RGC-fibroblast/MDCK interaction. PP2, PP3 and STI571 were added to the bathing medium 20 min prior to addition of ephrin-A5-Fc or Sema3A, and 20-30 min prior to RGC-fibroblast/MDCK interaction. STI571 was added to the bathing medium of COS cells transfected with EphA4 for 2 hours prior to cell lysis (see below). Eph-A5-Fc dimers (R & D Systems) were added at a final concentration of $1\mu\text{gml}^{-1}$ 10-20 min prior to RGC-fibroblast/MDCK interaction.

Table 2.3 Pharmacological inhibitors

Inhibitor (Target)	Final Conc.	Carrier	Reference (Source)
Y27632 (ROCK)	10 μM	H ₂ O	Ishizaki et al., 2000; Uehata et al., 1997. (Calbiochem)
PP2 (Src family kinases)	10 μM	DMSO	Hanke et al., 1996. (CN Biosciences)
PP3 (negative control PP2)	10 μM	DMSO	Traxler et al., 1996. (CN Biosciences)
STI571 (Abl family kinases)	10 μM	DMSO	Buchdunger et al., 1996; Drucker et al., 1996. (Novartis)

2.9. Biochemical techniques

2.9.1. Cell lysis

Isolated RGCs treated with clustered ephrin-A5-Fc, or COS cells transfected with EphA4, were placed on ice, the medium aspirated off and the cells washed 4 times with cold Tris-buffered saline (TBS). After the final wash had been removed 200 μl of cold RIPA buffer was added to each dish of cells. The lysed cells were collected at the edge of the dish using a cell scraper (Marathon) and passed 3 times through a

23G microlance needle (H M & S). The lysed cells were transferred to a 1.5 ml centrifuge tube (Eppendorf) on ice and spun at 5°C and 13000 rpm for 5 min. The supernatant was transferred to a clean 1.5 ml centrifuge tube on ice and mixed with an equal volume of 2x Laemmli sample buffer (Sigma).

2.9.2. Immuno-precipitation.

COS cells transfected with EphA4 were placed on ice, the medium aspirated off and the cells washed 4 times with cold TBS. After the final wash had been removed 100µl of cold immuno-precipitation (IP) buffer was added to each well. The lysed cells were collected at the edge of the dish using a cell scraper and passed 3 times through a 23G microlance needle. The lysed cells were transferred to a 1.5 ml centrifuge tube on ice and spun at 5°C and 13000 rpm for 5 min. The supernatant was transferred to a clean 1.5 ml centrifuge tube on ice. 20µl was removed for analysis of proteins by western blot, added to an equal volume of 2x Laemmli sample buffer and incubated in a heat block at 95°C for 5 min. The remaining cell lysate was incubated overnight at 5°C with 2µl anti-Mena antibody (BD biosciences) or 2.5µl anti-c-abl antibody (Merk Biosciences) on a rotating wheel.

Protein G agarose beads (Upstate Biotech) were washed 4 times by addition of IP buffer and spinning at 2000 rpm for 20 seconds), taking care to keep the volume constant. 50µl of equilibrated protein G agarose was added to each sample of immuno-precipitate and incubated for a further hour on a rotating wheel at 4°C. The protein G agarose was washed 4 times with IP buffer as above, and then all the supernatant was removed. 25µl of 2x Laemmli sample buffer was added and the samples incubated at 95°C for 5 min. The samples were immediately analysed by western blotting.

2.9.3. SDS-PAGE and Western blotting.

10-15% SDS-polyacrylamide gels were poured by hand using the Bio-Rad Mini-Gel system (Bio-Rad) and 10-20µL of lysate or immuno-precipitated sample was loaded to each well. The samples were separated by SDS-PAGE at 150V for 90-120 min and transferred to PVDF membrane (Amersham) using the Protean 3 electrophoresis system (Bio-Rad). Transfer buffer was used cold, and protein transfer was carried out at 4°C overnight. The membranes were blocked in a 1% solution of Superblock

(Pierce) in TBS-Tween (TBST) for 90 min at 4°C on an orbital shaker. Primary antibodies were diluted in 1% Superblock-TBST and membranes were incubated with primary antibody at room temperature for 30-45 min. The membranes were rinsed 3 times in TBST and then washed for 20-30 min with a minimum of 3 changes of TBST. Secondary antibodies were diluted in 0.1% Superblock-TBST and membranes incubated with secondary antibody for 30-40 min at room temperature. Membranes were rinsed and washed in TBST for 45-60 min, and the secondary antibodies visualised with SuperSignal West Pico chemiluminescent ECL substrate and CL-Xposure film (both Pierce).

Table 2.4. Antibodies used for Western blotting.

Antibody	Source (Clone)	Dilution	Source
Phospho-Eph receptor	Rabbit	1:10000	
Alpha tubulin	Rat (YL1/2)	1:2500	Serotec
Mena	Mouse (21)	1:250	BD Biosciences
c-Abl	Mouse (Ab-3)	1:50	Oncogene
Phospho-tyrosine	Mouse (PT66)	1:1000	Sigma
HRP-anti-rabbit	Goat	1:20000	Stratech
HRP-anti-rat	Donkey	1:50000	Stratech
HRP-anti-mouse	Goat	1:20000	Stratech

Chapter 3. Contact-induced RGC repulsive responses mediated by EphA signalling.

3.1. Introduction.

During development of the visual system RGC axons project to positions in their midbrain target (the tectum in avians, amphibians and fish, the superior colliculus [SC] in mammals) in a spatially organised manner, to produce a topographic map. Specifically axons arising from RGCs in the temporal retina terminate in the anterior tectum, while nasal RGC axons project to the more posterior tectum/SC (Holt and Harris, 1993; Mey and Thanos, 1992). As discussed in section 1.3.1 there is to date a large body of evidence that the topographic mapping that occurs along the anterior-posterior axis during the development of the retinotectal projection is governed by an increasing anterior to posterior gradient of ephrin-As expressed in the tectum/SC, and that these ephrin-As guide RGCs, which express a corresponding high temporal to low nasal gradient of EphA receptors, by differential repulsion (Kullander and Klein, 2002; Wilkinson, 2001).

Traditionally axon guidance directed by repulsive guidance cues has been studied *in vitro* using the related phenomenon of growth cone collapse (Cox et al., 1990; Raper and Kapfhammer, 1990; Walter et al., 1990a). For example addition of a suspension of membranes isolated from the chick tectum to the bathing medium of RGCs in culture induces the loss of the F-actin-rich lamella and filopodia that comprise the growth cone, which is present at the tip of an extending axon (Cox et al., 1990). Since the cloning of ephrin-A5 and ephrin-A2 the growth cone collapse assay has been routinely used to describe the differential repulsive response of temporal and nasal RGC axons to ephrin-As, and to identify potential signalling intermediates involved in the RGC response to EphA receptor activation (Cheng et al., 2003; Journey et al., 2002; Menzel et al., 2001; Monschau et al., 1997; Shamah et al., 2001; Wahl et al., 2000). The growth cone collapse assay used in these studies involves

addition of chimeric proteins, comprising the extracellular receptor-binding domain of ephrin-A fused to the Fc fragment of human IgG or alkaline phosphatase in order that they are soluble, to the bathing medium of RGCs *in vitro*. Ephrin-As are membrane bound *in vivo* and therefore guide axons by contact repulsion rather than acting as soluble, diffusible guidance cues (Gale et al., 1996b; Orioli and Klein, 1997; Pandey et al., 1995a; Tessier-Lavigne and Goodman, 1996; Wilkinson, 2001). A more physiologically relevant assay with which to study the RGC response to EphA receptor activation would therefore involve contact between a RGC growth cone and a cell that expresses endogenous levels of ephrin-A.

A series of elegant studies by Roger Davenport and his colleagues have described the response of RGCs to contact with isolated tectal cells, but these studies do not address whether the RGC response is due to EphA receptor signalling (Davenport et al., 1996; Davenport et al., 1998; Davenport et al., 1999; Thies and Davenport, 2003). In order to develop a physiologically relevant assay with which to study the neuronal response to EphA receptor activation I have described the response of nasal RGCs *in vitro* to contact with individual Swiss-3T3 fibroblasts, which express endogenous levels of ephrin-A on their surface. In response to contact with an individual Swiss-3T3 fibroblast RGCs exhibit rapid loss of growth cone lamella followed by axon retraction. Contact with a Swiss-3T3 fibroblast induces activation of Eph receptors on the RGC growth cone, and disruption of the EphA-ephrin-A interaction prevents the contact-induced loss of RGC growth cone lamella and axon retraction. Together with the observation that overexpression of ephrin-A5 in a cell type that does not endogenously express ephrin-A is sufficient to induce the RGC repulsive response, these data provide strong evidence that the robust and reproducible RGC response to contact with a Swiss-3T3 fibroblast described in this chapter is mediated by neuronal EphA signalling, which justifies the use of this co-culture assay to investigate signalling intermediates involved in EphA signalling in RGCs.

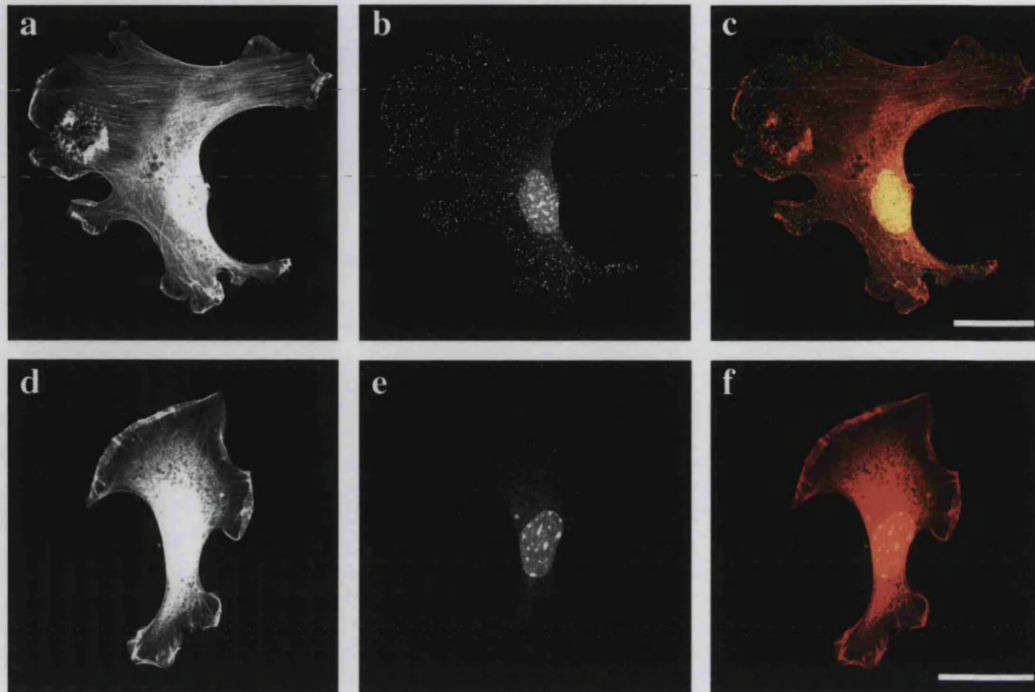
3.2. Results.

3.2.1. Swiss-3T3 fibroblasts endogenously express ephrin-A.

Roger Davenport and colleagues have previously described the response of individual RGCs to contact with individual primary tectal neurons that express ephrin-As (Davenport et al., 1996; Davenport et al., 1998; Davenport et al., 1999; Thies and Davenport, 2003). Given the steep ephrin-A expression gradient along the anterior-posterior axis of the tectum it is unlikely that, even within a sub-population of tectal cells, the level of ephrin-A expression is uniform (Cheng et al., 1995; Drescher et al., 1995; Monschau et al., 1997), and this could lead to a variable RGC response to contact with a tectal neuron. In addition tectal neurons in culture have very round cell bodies, making careful analysis of the behaviour of RGC growth cone filopodia and lamellæ at the sites of cell-cell contact difficult when viewed under phase time-lapse microscopy. In order to develop a reproducible assay with which to study the response of RGC growth cones to contact with an ephrin-A-expressing cell, I have screened a variety of cell lines for endogenous, uniform ephrin-A expression, and a flat, well-spread morphology.

Swiss-3T3 fibroblasts, when plated at low density on laminin and grown in RGC medium, are flat cells with large spread lamellae (Fig 3.1a) and therefore have suitable morphology to allow study of contact-induced responses in RGC growth cones. Cell surface ephrin-A expression can be detected by indirect immunofluorescence using a chimeric protein composed of the extracellular ligand-binding domain of EphA5, which has selective binding affinity for ephrin-As (Davis et al., 1994; Gale et al., 1996a; Gale et al., 1996b), fused to the Fc fragment of human IgG (EphA5-Fc) (Davy et al., 1999; Krull et al., 1997; Marcus et al., 2000). EphA5-Fc, added to the bathing medium of live Swiss-3T3 fibroblasts in culture, can be visualised after fixation with a fluorescently-tagged secondary antibody, revealing clusters of ephrin-A evenly distributed across the entire surface of the fibroblast under conditions in which it can be co-cultured with RGCs (Fig 3.1b and green in 3.1c). Ephrin-A distribution extends to the edge of the Swiss-3T3 lamella (Fig 3.1c), indicating that contact of a RGC growth cone with a Swiss-3T3 fibroblast should lead to activation of EphA receptors on the RGC growth cone regardless of the site of contact. It should be noted that EphA5-Fc does not detect ephrin-A on the surface

Figure 3.1. Swiss-3T3 fibroblasts in culture express endogenous ephrin-A.



Under conditions in which they can be co-cultured with RGCs, Swiss-3T3 fibroblasts are flat with spread lamellae, shown by fluorescent phalloidin staining (a and d). Detection of surface ephrin-As using EphA5-Fc, added to live Swiss-3T3 fibroblasts (a-c), and a fluorescent secondary antibody (b, e, and green in c and f) reveals evenly distributed clusters of ephrin-A across the Swiss-3T3 fibroblast surface. Omitting EphA5-Fc (d-f) acts as a control for non-specific antibody staining. Scale bars = 20 μ m.

c and f are merged images.

of the Swiss-3T3 fibroblast unless it is clustered into oligomers with an anti-human antibody before being added to the bathing medium. This may account for the punctuate staining shown in Fig 3.1b and Fig 3.1c, and it is possible that under basal conditions ephrin-As are present in a more diffuse pattern on the surface of a Swiss-3T3 fibroblast in culture. Fig 3.1d-f are included to control for non-specific antibody binding.

I attempted to characterise the particular ephrin-A family members expressed by Swiss-3T3 fibroblasts using Western blot and by immunofluorescence. Unfortunately the commercially available antibodies raised against individual ephrin-A family members failed to produce a detectable signal by either method. It is not clear that identifying the particular ephrin-A members expressed by Swiss-3T3 fibroblasts will provide additional evidence that these cells are suitable with which to investigate the behaviour of RGCs, beyond the observation reported here that Swiss-3T3 fibroblasts show ephrin-As evenly distributed across their surface. It has been reported that the EphA receptors expressed on RGCs have up to a ten-fold difference in binding affinities for different members of the ephrin-A family, but these experiments were performed using soluble-A proteins, whose affinity for their receptor alone reflects whether binding will occur (Flanagan and Vanderhaeghen, 1998; Gale and Yancopoulos, 1997). This does not necessarily reflect the biological relevance of interaction at the cell-cell interface, where co-operative interactions between multiple Eph receptor-ephrin family members simultaneously may influence the binding between particular partners (Gale and Yancopoulos, 1997). For the same reason, experiments that report nasal RGCs *in vitro* show a repulsive response to ephrin-A5, but not ephrin-A2 or ephrin-A6 use only one ligand, and might not reflect the physiological situation (Menzel et al., 2001; Monschau et al., 1997). The uniform distribution pattern of ephrin-A across the surface of Swiss-3T3 fibroblasts (Fig 3.1) will allow detailed analysis of the dynamic RGC behaviour of a RGC at the site of contact with a cell that expresses endogenous levels of ephrin-A.

3.2.2. Contact with an ephrin-A-expressing Swiss-3T3 fibroblast induces a rapid repulsive response in nasal RGCs.

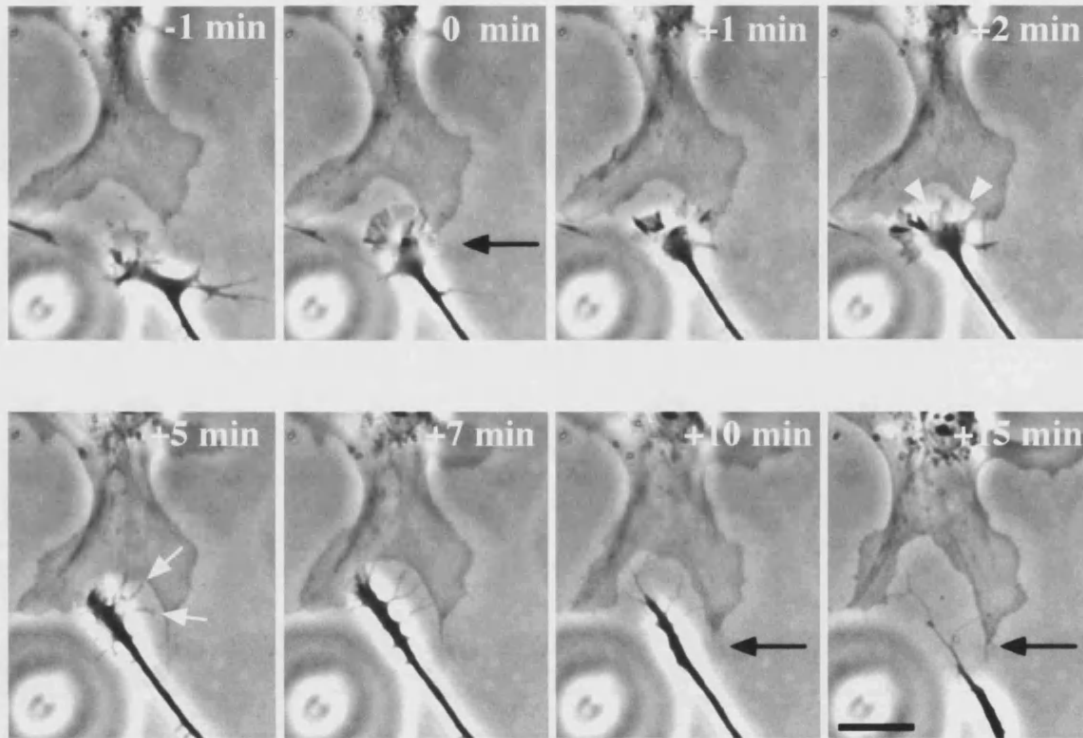
In order to describe the dynamic response of a single RGC growth cone to contact with an ephrin-A-expressing Swiss-3T3 fibroblast, interactions between the two cell

types were recorded by phase time-lapse microscopy. An example of the repulsive response induced in a RGC following contact with a Swiss-3T3 fibroblast is shown in Fig 3.2 (See also Movie 3.2 on the enclosed CD). The RGC growth cone is highly dynamic prior to contact with the ephrin-A-expressing fibroblast, and within 2 min of contact the RGC lamella begins to retract from the site of cell-cell contact (Fig 3.2, arrowheads, Movie 3.2). The RGC growth cone lamella is completely lost by 7 min after contact. Loss of RGC growth cone lamella is assessed under the strict criteria of complete absence of lamellæ for longer than 1 min (see section 2.7.1). The majority (76.7%) of RGCs completely lose growth cone lamellæ within 10 min of initial contact with an ephrin-A-expressing Swiss-3T3 fibroblast (Table 3.1). Of the RGCs that lose growth cone lamellæ within the normal period of recording (25 min after contact), on average the lamella is completely lost by 5.9 ± 2.5 min (n=25).

After the RGC growth cone is completely lost the RGC axon begins to retract from the site of cell-cell contact (Fig 3.2). Axon retraction is defined as the phase-dark central zone of the growth cone, revealed as the axon terminal when the lamella is lost, retracting beyond its position at the time of initial contact (Fig 3.2 black arrows), and by these criteria 43.3% of RGC axons retract within 20 min of initial contact with a Swiss-3T3 fibroblast (Table 3.1). In the example shown in Fig 3.2 axon retraction occurs at 10.5 min after contact and by 15 min the axon has retracted well beyond the point of initial contact. The RGC repulsive response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast consists of a series of distinct events. In the majority (69.2%) of RGC-Swiss-3T3 fibroblast interactions the lamella is completely lost before the axon begins to retract, and on average axon retraction begins at 8.3 ± 4.8 min after initial contact (n=13).

As the growth cone lamella is lost following contact with a Swiss-3T3 fibroblast, the growth cone filopodia are revealed (Fig 3.2 white arrows). Most of these filopodia are pulled away as the axon retracts, leaving behind very few, fine retraction fibres. A negative correlation exists between the presence of growth cone filopodia, revealed as the lamella is lost, and axon retraction. Of the 10 RGCs that lose lamella but do not retract following contact with a Swiss-3T3 fibroblast, 9 retain more than 3 filopodia after complete loss of growth cone lamellæ. In comparison, of the RGC growth cones that completely lose lamella before retracting (n=9) 8 leave behind 3 or fewer retraction fibres, which appear to have initially been growth cone filopodia, 1

Figure 3.2. Contact with an ephrin-A-expressing fibroblast induces loss of RGC growth cone lamella and axon retraction.



Time-lapse stills showing that contact with a Swiss-3T3 fibroblast induces a rapid repulsive response in an individual nasal RGC axon. Following contact between the RGC growth cone lamella and the Swiss-3T3 fibroblast the growth cone lamella begins to collapse (arrowheads). As the growth cone lamella is lost, growth cone filopodia are revealed (white arrows). After the growth cone lamella is completely lost, the RGC axon begins to retract, and by 15 min after initial contact has retracted well beyond its position at the time of initial contact (black arrows). As the RGC axon retracts most of the growth cone filopodia are pulled away, leaving behind only a few, very fine retraction fibres. Times shown are relative to initial contact. Scale bar = 20 μ m.

Table 3.1.

Cell type and treatment (n)	RGCs showing loss of lamella within 10 min of contact.	RGCs showing axon retraction within 20 min of contact.
Swiss 3T3 fibroblast (30)	76.7%	43.3%
Swiss 3T3 fibroblast + EphA5-Fc (16)	6.3%	0.0%
MDCK (15)	26.7%	6.7%
MDCKephrin-A5 (16)	87.5%	75%
MDCK mock (4)	25.0%	25.0%

Table 3.2.

Cell type and treatment (n)	Contacting cells withdrawing >10µm within 10 min of contact with a RGC.
Swiss 3T3 fibroblast (21)	52.4%
Swiss 3T3 fibroblast +EphA5-Fc (11)	27.2%
MDCK (8)	50.0%
MDCK-ephrin-A5 (9)	0.0%
MDCK-mock (4)	33.3%

minute after axon retraction begins, supporting the idea that filopodia are pulled away by the retracting axon. Phase time-lapse microscopy of RGC-Swiss-3T3 fibroblast interactions therefore reveals that contact with an ephrin-A-expressing Swiss-3T3 fibroblast induces a repulsive response in the RGC, which consists of a sequence of distinct cellular events, each of which can be quantified.

Interestingly the Swiss-3T3 fibroblast also responds to contact with a RGC growth cone; withdrawing its lamella at the site of cell-cell contact (Fig 3.2). Approximately half (52.4%) of Swiss-3T3 fibroblasts withdraw further than 10 μ m from the site of initial cell-cell contact within 10 min (n=21, see Table 3.2), but this response does not appear to be the result of EphA-ephrin-A interaction (see below).

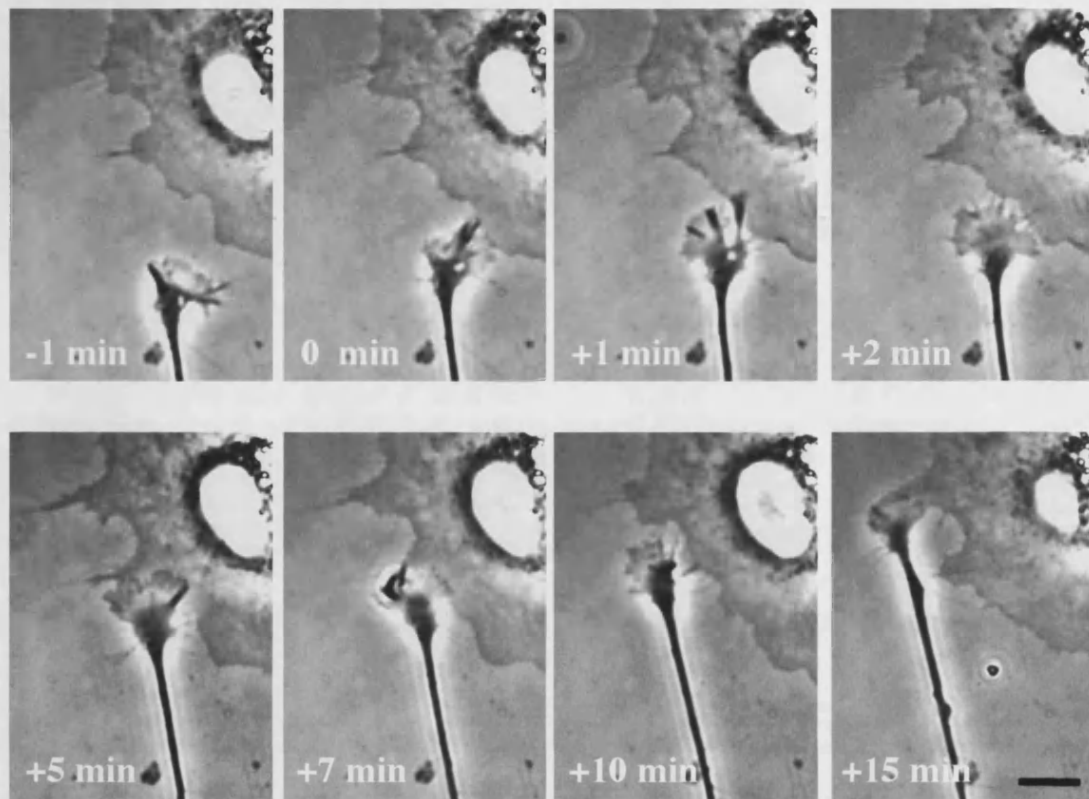
3.2.3. Blocking EphA-ephrin-A interaction prevents the RGC repulsive response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast.

It is clear that contact with an ephrin-A-expressing Swiss-3T3 fibroblast results in a robust and reproducible RGC response. To make use of the contact-induced RGC response as an assay with which to investigate signalling pathways downstream of EphA receptor activation, the loss of RGC growth cone lamellæ and axon retraction must depend on Eph receptor-ephrin interaction. Soluble EphA5-Fc, presented as unclustered dimers, has previously been used to disrupt potential EphA-ephrin-A interactions *in vitro*, to demonstrate that the inhibitory response of mouse RGCs to ephrin-A-expressing tissue from the optic chiasm is mediated by EphA signalling (Marcus et al., 2000). In order to demonstrate that the RGC response to contact with a Swiss-3T3 fibroblast is due to Eph receptor-ephrin interaction, 1 μ gml⁻¹ EphA5-Fc was added to the bathing medium of RGCs and Swiss-3T3 fibroblasts in co-culture 10-20 min prior to cell-cell contact. EphA5 has a high affinity for all ephrin-As, but does not bind ephrin-Bs (Davis et al., 1994; Gale et al., 1996a; Gale et al., 1996b; Monschau et al., 1997). Soluble dimers of the extra-cellular binding domain of EphA5 retain the ability to bind ephrin-As (Monschau et al., 1997) but are not clustered into oligomers considered necessary to activate reverse signalling (Huai and Drescher, 2001). EphA5-Fc dimers added to the bathing medium will therefore bind to and mask ephrin-As on the surface of the Swiss-3T3 fibroblast, preventing their interaction with EphA receptors on the RGC growth cone.

An example of the interaction between a RGC growth cone and a Swiss-3T3 fibroblast in the presence of EphA5-Fc is shown in Fig 3.3^{*}. Following contact with the Swiss-3T3 fibroblast, the RGC growth cone lamella does not collapse. Instead the RGC growth cone continues to advance across the substrate, lamella intact, for more than 15 min after initial contact with the Swiss-3T3 fibroblast. Addition of EphA5 prior to cell-cell contact significantly reduces the percentage of RGCs that completely lose lamellæ following contact with an ephrin-A-expressing Swiss-3T3 fibroblast (6.3%, see Table 3.1) compared to control interactions (76.7%, $p < 0.0001$, Fisher's exact test for independence). In addition, the presence of EphA5-Fc completely abolishes the RGC axon retraction in response to contact with an ephrin-A expressing fibroblast. Of the 16 RGC-Swiss-3T3 interactions recorded in the presence of EphA5-Fc, none resulted in RGC axon retraction, compared to 43.3% of control interactions ($p < 0.002$, Fisher's exact test for independence, See Table 3.1). In contrast EphA5-Fc does not significantly reduce the percentage of Swiss-3T3 fibroblasts that withdraw following contact with a RGC growth cone ($p > 0.2$, Fisher's exact test for independence, see Table 3.2), suggesting that the fibroblast response is not induced by EphA-ephrin-A interaction. Together the above data provide strong evidence that the loss of RGC lamellæ and axon retraction induced by contact with a Swiss-3T3 fibroblast are mediated by EphA-ephrin-A interaction.

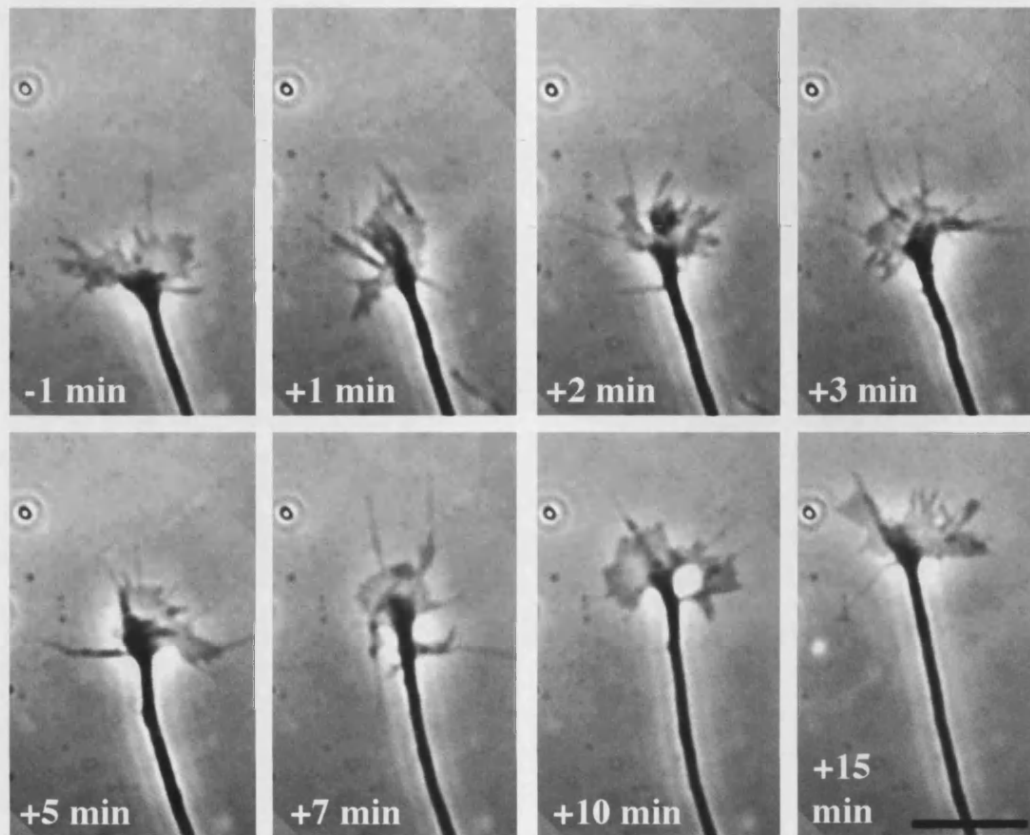
It is formally possible that the RGC repulsive response following contact with a Swiss-3T3 fibroblast is due to reverse signalling through the ephrin-As expressed on RGCs in culture (Hornberger et al., 1999). Swiss-3T3 fibroblasts in culture express low levels of EphA receptors (C.D. Nobes, unpublished observations) and therefore contact between a RGC growth cone and a Swiss-3T3 fibroblast may induce signalling through neuronal ephrin-As, rather than through EphA receptors. The observation that EphA5-Fc inhibits the RGC repulsive response does not distinguish between these possibilities. It has been reported that ephrin-A-mediated signalling in non-neuronal cells increases cell-matrix adhesion, and in primary neurons, induces neurite outgrowth (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). Activation of ephrin-A-signalling in RGCs would therefore not be predicted to induce RGC growth cone collapse and axon retraction, but to address this issue directly I have stimulated RGCs in culture with soluble EphA5-Fc, clustered into oligomers in order to activate ephrin-A reverse signals. Fig 3.4[†] clearly demonstrates

Figure 3.3. Blocking EphA-ephrin-A interaction prevents the RGC repulsive response to contact with a Swiss-3T3 fibroblast.



Time-lapse stills showing that disruption of EphA-ephrin-A interaction, using soluble EphA5-Fc, prevents both the loss of RGC lamella and axon retraction induced by contact with a Swiss-3T3 fibroblast. EphA5-Fc (1 μ g/ml) was added to the bathing medium of Swiss-3T3 fibroblasts and RGCs in co-culture 10-20 min prior to cell-cell contact. In the presence of EphA5-Fc the RGC growth cone continues to advance across the substrate, lamella intact, for more than 15 min after initial contact with the ephrin-A-expressing Swiss-3T3 fibroblast. Times shown are relative to initial contact. Scale bar = 20 μ m.

Figure 3.4. Activation of ephrin-A signalling does not induce growth cone collapse or axon retraction.



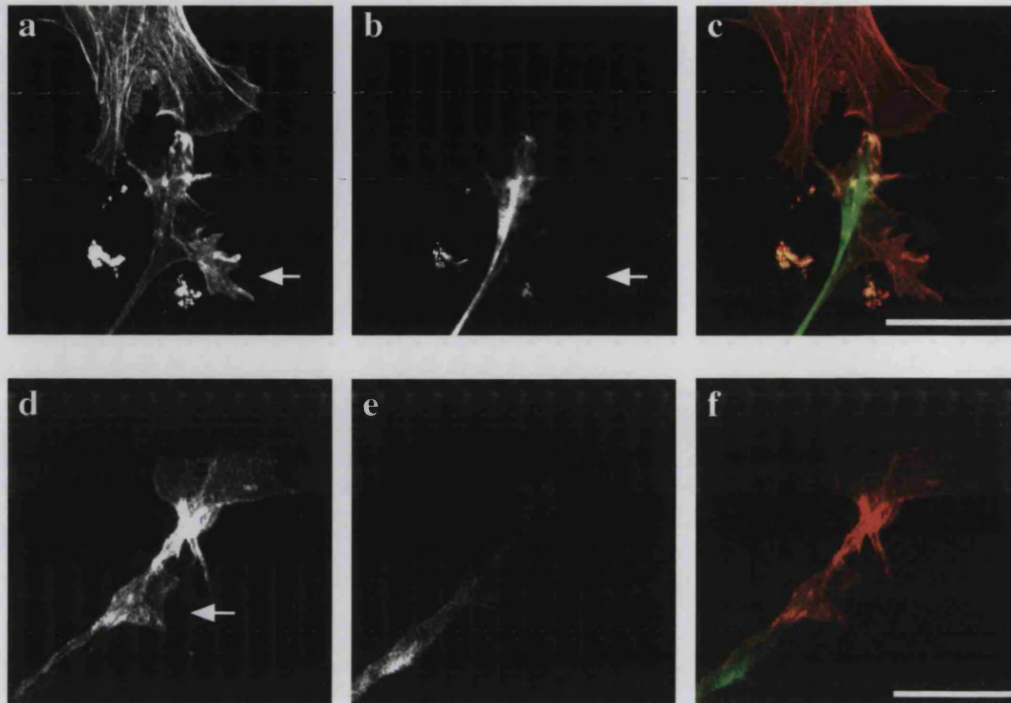
Time-lapse stills showing that stimulation of ephrin-A signalling in RGCs, by addition of soluble clustered EphA5-Fc, does not induce loss of RGC lamella, or axon retraction. Following addition of clustered EphA5-Fc (1 μ g/ml) the RGC continues to advance across the substrate, lamella intact. Times shown are relative to addition of EphA5-Fc. Scale bar = 20 μ m.

that stimulation with clustered EphA5-Fc does not induce RGC collapse and axon retraction. The RGC growth cone retains a full, spread lamella following addition of EphA5-Fc and the axon continues to advance across the substrate. Taken together the data described above provide strong evidence that the RGC repulsive response to contact with a Swiss-3T3 fibroblast is mediated by activation of neuronal EphA receptors following interaction with ephrin-As on the surface of a Swiss-3T3 fibroblast.

3.2.4. Following contact with a Swiss-3T3 fibroblast, Eph receptors on the RGC growth cone become activated.

Eph receptors contain a highly conserved pair of tyrosine residues in the juxtamembrane region that are major sites of autophosphorylation, and phosphorylation at these sites is required for ligand-activated Eph kinase activity (Binns et al., 2000; Choi and Park, 1999; Ellis et al., 1996; Fox et al., 1995; Kalo and Pasquale, 1999; Sajjadi and Pasquale, 1993; Wybenga-Groot et al., 2001; Zisch et al., 1998). Mutation of these residues in EphA4, which is expressed by nasal RGCs *in vitro* and is required for the repulsive response of RGCs to substrate-bound ephrin-A5, prevents EphA4 kinase activity against an exogenous substrate *in vitro* (Binns et al., 2000; Monschau et al., 1997; Walkenhorst et al., 2000). An antibody raised against Eph receptors phosphorylated on these residues (see section 2.4) can therefore be used as a read-out of Eph receptor activity (Marston et al., 2003; Shamah et al., 2001). Fig 3.5 shows that contact with a Swiss-3T3 fibroblast induces activation of Eph receptors on the RGC growth cone, particularly in the central region of the growth cone (Fig 3.5a-c). The experiment shown in Fig 3.5 was not done in such a way as to allow exact timing of the RGC-Swiss-3T3 fibroblast interaction, and it is possible that at earlier time-points Eph receptor activity is concentrated at the growth cone periphery. Note the growth cone of a fasciculated axon does not show Eph receptor phosphorylation (Fig 3.5 a, b, arrows). Interestingly there is a high level of Eph receptor phosphorylation along the RGC axon (Fig 3.5), which may be a result of axon fasciculation. RGCs cultured in the absence of Swiss-3T3 fibroblasts (Fig 3.5d-f) do not show any Eph receptor phosphorylation on the growth cone, or along the portion of the axon that is not in contact with another axon (Fig 3.5e and green in f). Beyond the fasciculated growth

Figure 3.5. Contact with a Swiss-3T3 fibroblasts induces activation of Eph receptors on the RGC growth cone.



RGCs, in the presence (a-c) or absence (d-f) of Swiss-3T3 fibroblasts, were fixed and stained with phalloidin (a and d, red in c and f) and anti-phospho-Eph receptor (b and e, green in c and f). A RGC growth cone in contact with a Swiss-3T3 fibroblast stains positive for phosphorylated Eph receptor (b), whereas the growth cone of a fasciculated RGC does not (a, b, arrows). RGC growth cones in the absence of Swiss-3T3 fibroblasts do not display phospho-Eph receptor staining (d-f). Note that fasciculation of RGC axons correlates with Eph receptor phosphorylation (a-c, d-f beyond the fasciculated growth cone {d, arrow}). Scale bars = 20 μ m.

c and f are merged images. It should be noted, as mentioned in section 2.4, the anti-phospho-Eph receptor antibody used was not affinity purified against the phosphopeptide, and therefore the staining pattern shown here may also reflect increased phosphorylation of other proteins.

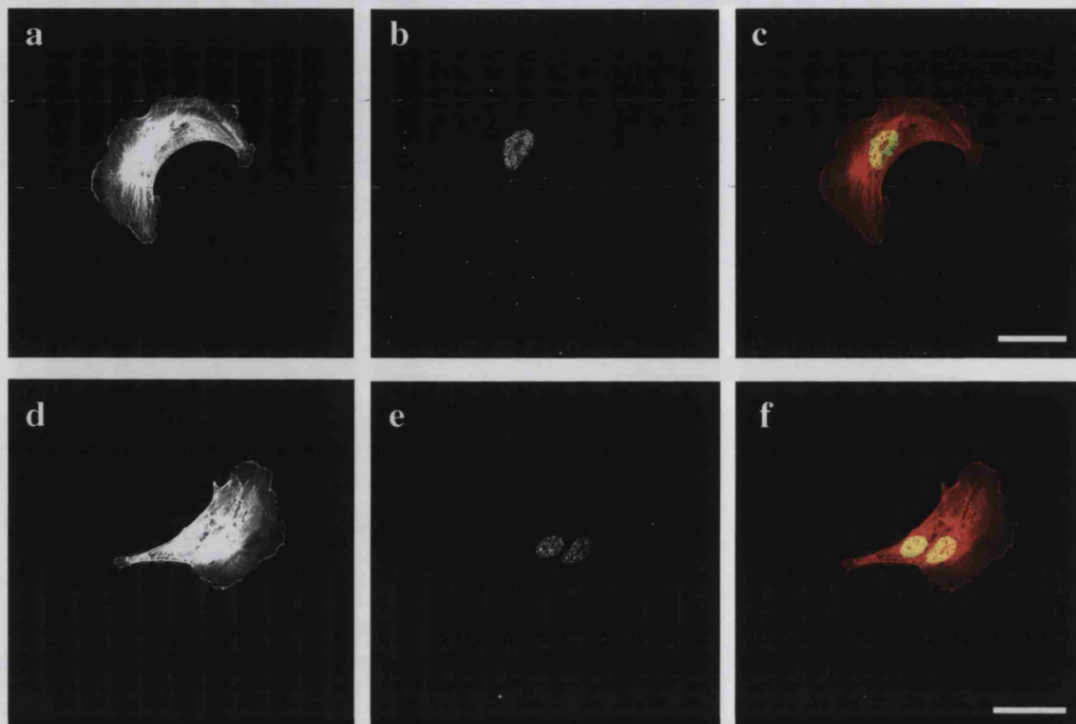
cone (Fig 3.5d arrow) however, the RGC axons are positive for phospho-Eph receptor (Fig 3.5d-f). EphA4 is highly phosphorylated in the nasal retina *in vivo*, and this is thought to be the result of co-expression of ephrin-A ligands by nasal RGCs (Connor et al., 1998; Hornberger et al., 1999). This would explain the fact that fasciculated nasal RGCs express phosphorylated Eph receptors (Fig 3.5). Importantly, phosphorylation of Eph receptors on the RGC growth cone is only seen following contact with an ephrin-A-expressing Swiss-3T3 fibroblast (Fig 3.5), which provides further evidence that the repulsive RGC response induced by contact with these cells is mediated by Eph receptor activation.

3.2.5. MDCK cells do not express endogenous ephrin-A and induce a much-reduced RGC repulsive response.

The observation that blocking EphA-ephrin-A interaction prevents loss of RGC lamellæ and axon retraction suggests that ephrin-A expression on the contacting cell is necessary for contact-induced loss of RGC lamellæ and axon retraction. In order to test this hypothesis I have investigated the RGC response to contact with a cell type that does not endogenously express ephrin-As. Sub-confluent MDCK cells plated on laminin and grown in RGC medium have a similar actin morphology to sub-confluent Swiss-3T3 fibroblasts, in that they are well spread with large lamellæ (Fig 3.6a, d). However under these conditions MDCK cells do not display detectable amounts of ephrin-A on their surface by indirect immunofluorescence using EphA5-Fc (Fig 3.6b, e, green in 3.6c, f). Recombinant rat ephrin-A1-Fc has previously been used to demonstrate that MDCK cells in culture endogenously express EphA receptors (Miao et al., 2003), ruling out the possibility that the recombinant rat EphA5-Fc used to detect surface ephrin-A in Fig 3.6 does not bind canine ephrin-A.

Fig 3.7 shows an example of the interaction between a RGC growth cone and an MDCK cell. Following contact with the MDCK cell the RGC growth cone does not lose lamella but begins to spread intact lamella along the edge of the MDCK cell. At later time-points (7, 10 and 15 min) after the initial contact the RGC growth cone lamella becomes spread over a large area, which is a prelude to this particular growth cone bifurcating as the axon branches (Movie 3.7). By 20 min after contact a smaller growth cone is present on the left-hand axon branch (Fig 3.7B arrow), while the right-hand branch has grown under the MDCK cell (Fig 3.7B arrowhead). The

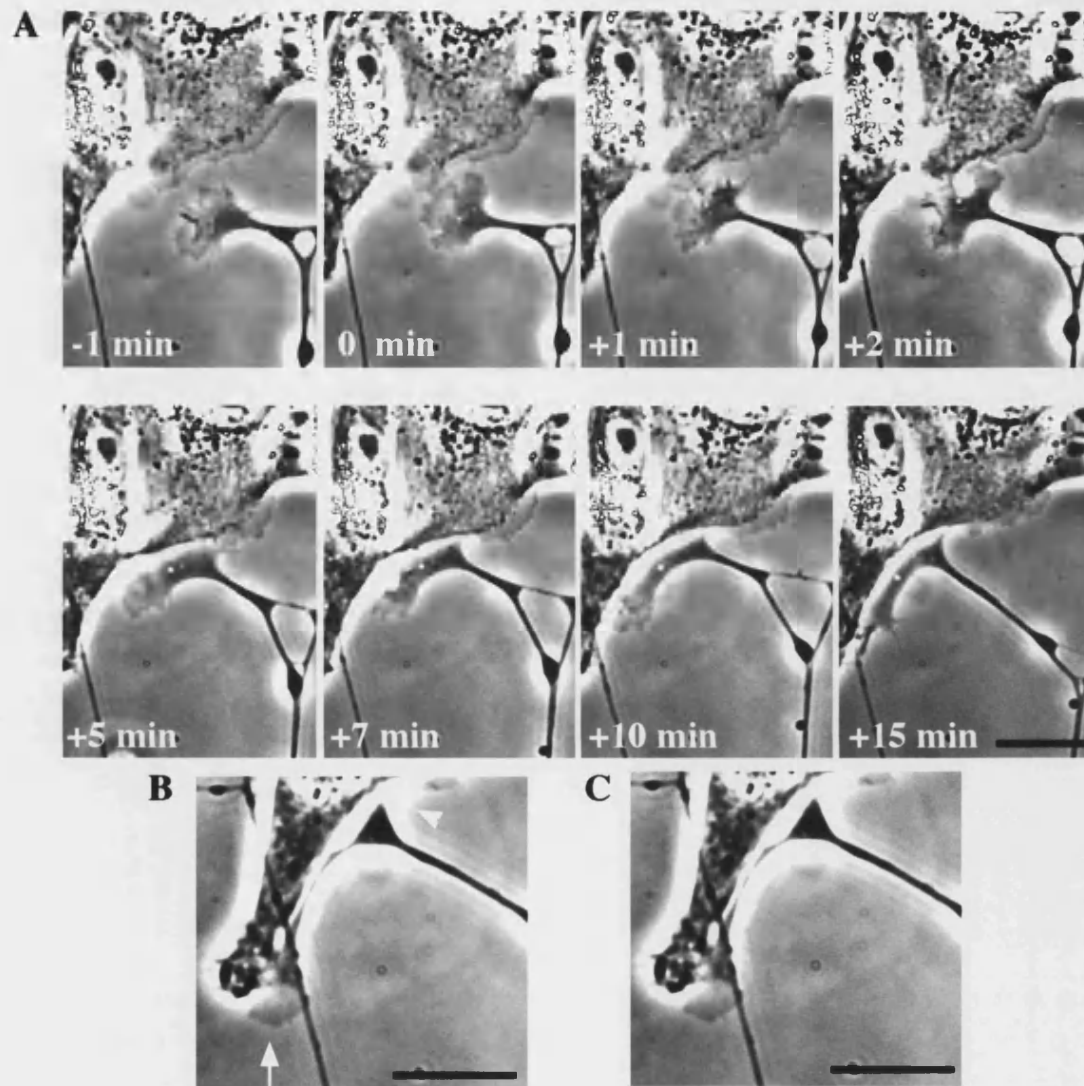
Figure 3.6. MDCK cells in culture do not express endogenous ephrin-A.



Under conditions in which they can be co-cultured with RGCs, MDCK cells are flat with spread lamellae, shown by fluorescent phalloidin staining (a and d, red in c and f). EphA5-Fc, added to live MDCK cells (a-c), and a fluorescent secondary antibody (b, e, and green in c and f) does not reveal any surface ephrin-A staining compared with the surface ephrin-A staining seen on Swiss-3T3 fibroblasts (Fig 3.1). Omitting EphA5-Fc (d-f) acts as a control for non-specific antibody staining. Scale bars = 20 μ m.

c and f are merged images.

Figure 3.7. Contact with an MDCK cell does not induce loss of RGC growth cone lamella or axon retraction.



A. Time-lapse stills showing that interaction with an MDCK cell, which does not express detectable levels of ephrin-A, does not induce loss of growth cone lamella or axon retraction in a contacting RGC. The RGC growth cone grows around the edge of the MDCK cell without losing its lamella for more than 15 min after contact. After the RGC growth cone has split, the right hand branch is not visible, as it has grown under the MDCK cell (**B**, arrowhead). The left hand axon branch has a growth cone that continues to grow along the edge of the MDCK cell (**B**, arrow), retaining its lamella for the entire period of filming (**C**, 25 min after initial contact). Times shown are relative to initial contact. Scale bars = 20 μ m.

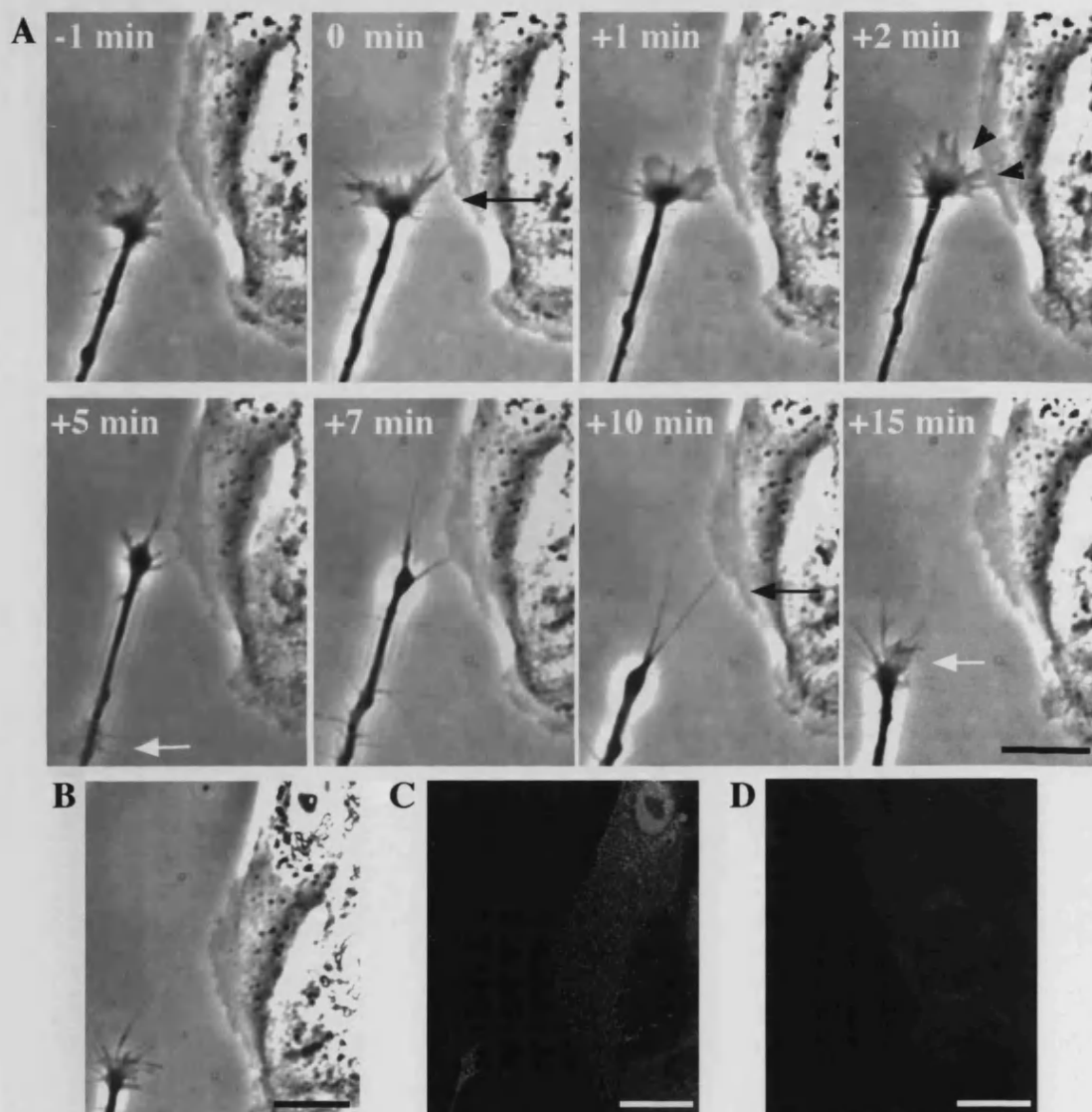
newly visible growth cone continues to advance along the edge of the MDCK cell, lamella intact, for the remainder of the recording (25 min after initial contact, Fig 3.7C). Analysis of 15 RGC-MDCK interactions reveals that only 26.7% of RGC growth cones completely lose lamellæ within 10 min of contact with an MDCK cell (table 3.1) compared with 76.7% of RGCs contacting a Swiss-3T3 fibroblast ($p < 0.003$, Fisher's exact test for independence). In addition a very low percentage of RGC axons retract following contact with an MDCK cell (6.7%, see table 3.1) compared with a Swiss-3T3 fibroblast (43.3%, $p < 0.02$, Fisher's exact test for independence). This greatly reduced repulsive response correlates with lack of detectable ephrin-A expression and therefore the above data demonstrate that contact with a cell type that does not endogenously express ephrin-A results in a significantly reduced RGC repulsive response compared to contact with an ephrin-A-expressing cell.

3.2.6. Expression of ephrin-A5 in MDCK cells is sufficient to induce a RGC repulsive response.

Ephrin-A2 and ephrin-A5 appear to be the major members of the ephrin-A family that confer topographic specificity along the anterior-posterior axis *in vivo* (Feldheim et al., 2000; Frisen et al., 1998). Nasal RGCs *in vitro* are insensitive to ephrin-A2, but show repulsive responses to soluble and substrate-bound ephrin-A5 (Monschau et al., 1997; Nakamoto et al., 1996). In order to demonstrate that ephrin-A-expression in the contacting cell is sufficient to induce loss of RGC lamella and axon retraction I have microinjected MDCK cells with a mammalian expression vector encoding ephrin-A5 (MDCKephrin-A5 cells), along with FITC-dextran as an injection marker. Allowing 3-4 hours for expression of ephrin-A5, injected MDCK cells were selected for their proximity to an advancing RGC growth cone, and the resulting axon retraction was observed by time-lapse microscopy.

An example of the interaction between a RGC growth cone and an MDCKephrin-A5 cell is shown in Fig 3.8. The RGC growth cone lamella begins to retract 2 min after initial contact with the MDCKephrin-A5 cell (Fig 3.8A arrowheads) and the lamella is completely lost by 5 min. Once the lamella is lost the RGC axon begins to retract and by 10 min after initial contact has retracted well beyond its position at the time of initial contact (Fig 3.8A black arrows). By 15 min the RGC axon has retracted

Figure 3.8. Expression of ephrin-A5 in MDCK cells is sufficient to induce loss of RGC growth cone lamella and axon retraction.



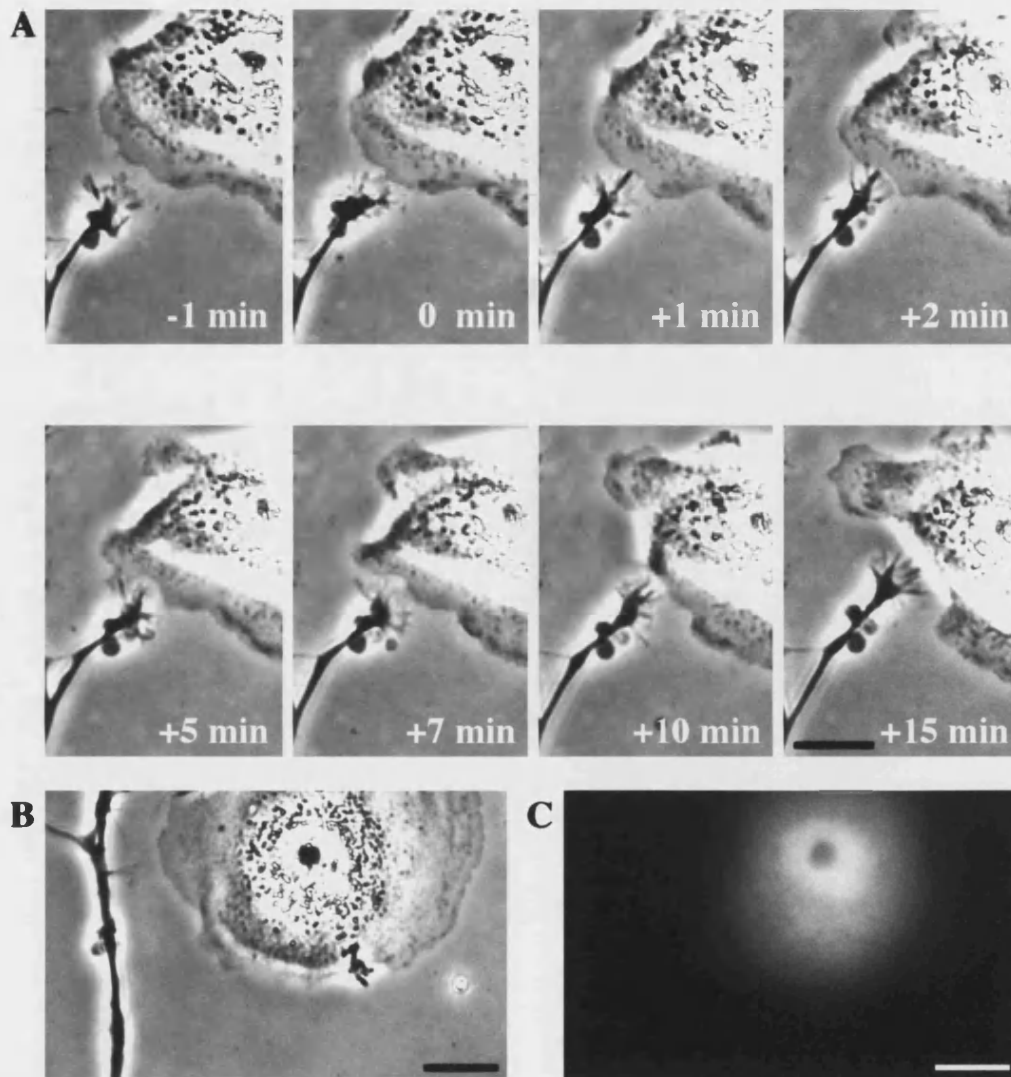
A. Time-lapse stills showing that contact with an ephrin-A5-expressing MDCK cell induces a rapid repulsive response in an individual nasal RGC axon. The growth cone lamella begins to collapse 2 min after initial contact with an MDCK cell (arrowheads), and is completely lost after 5 min. By 10 min the RGC axon has retracted well beyond its position at the time of initial contact (black arrows) and at 15 min has retracted beyond the growth cone of a fasciculated axon (white arrows). **B.** Final time-lapse still of Movie 3.8, showing a lower magnification view of the MDCK-ephrin-A5 cell shown in A. **C.** The cells were fixed and stained with anti-ephrin-A5 prior to permeabilisation, to demonstrate that the MDCK-ephrin-A5 cell that induces the RGC response shown in A is injected with FITC dextran (green) and expresses surface ephrin-A5 (red). **D.** A non-injected MDCK cell on the same coverslip does not express surface ephrin-A5. Scale bars = 20µm.

beyond the growth cone of a fasciculated RGC axon (Fig 3.8A white arrows). Fig 3.8B shows the final still from Movie 3.8 at 16 min after initial contact. The cells were fixed within a few minutes of this final image, hence the images in Fig 3.8B and C are not precisely superimposable. The MDCK cell that induces rapid loss of RGC growth cone lamella and axon retraction (Fig 3.8A) expresses ephrin-A5 (red in Fig 3.8C). FITC-dextran was included as an injection marker (green in Fig 3.8C). Fig 3.8D shows non-injected MDCK cells on the same coverslip to control for non-specific antibody staining.

Analysis of 16 interactions between a RGC growth cone and an MDCK-ephrin-A5 cell reveal that 87.5% of RGC growth cones completely lose lamellæ within 10 min of contact (Table 3.1). This value is significantly higher than the percentage of RGCs that completely lose lamellæ within 10 min of contact with a non-injected MDCK cell ($p < 0.001$, Fisher's exact test for independence), and is not significantly different from the percentage of RGC growth cones that lose lamellæ in response to contact with a Swiss-3T3 fibroblast, which endogenously expresses ephrin-A ($p > 0.4$, Fisher's exact test for independence). The majority (75.0%) of RGC axons retract within 20 min of contact with an MDCK-ephrin-A5 cell (Table 3.1), significantly more than undergo axon retraction in response to a non-injected MDCK cell ($p < 0.0002$, Fisher's exact test for independence). Over-expression of ephrin-A in NIH3T3 fibroblasts has previously been shown to induce axon retraction in an identical percentage of nasal RGCs following contact *in vitro* (Davenport et al., 1998).

Fig 3.9 demonstrates that injection of an MDCK cell with vector alone (mock-injected MDCK) does not affect the response of a RGC axon following contact with the injected cell. The RGC growth cone retains lamella for over 15 min after initial contact, and the RGC axon does not retract (Fig 3.9A). The final still from Movie 3.9 is shown in Fig 3.9B. This movie was left to record for a long time; the final still was taken over an hour after initial contact and therefore the RGC axon has grown past the MDCK cell (Fig 3.9B). In order to demonstrate that the MDCK cell shown in Fig 3.9A is injected, a fluorescence image showing FITC dextran is shown in Fig 3.9C. The percentage of RGCs that lose lamellæ within 10 min of contact with a mock-injected MDCK cell (25.0%, see table 3.1) is not significantly different from the percentage of axons that lose lamella in response to a non-injected MDCK cell

Figure 3.9. Contact with a mock-injected MDCK cell does not induce a RGC repulsive response.



A. Time-lapse stills showing that contact with a mock-injected MDCK cell does not induce loss of RGC growth cone lamella or axon retraction. Following contact with a mock-injected MDCK cell the RGC growth cone continues to advance across the substrate, lamella intact. **B.** The final still from Movie 3.9, to correspond with the fluorescent image in **C**, demonstrating that the MDCK cell has been injected with FITC dextran. Scale bars = 20 μ m.

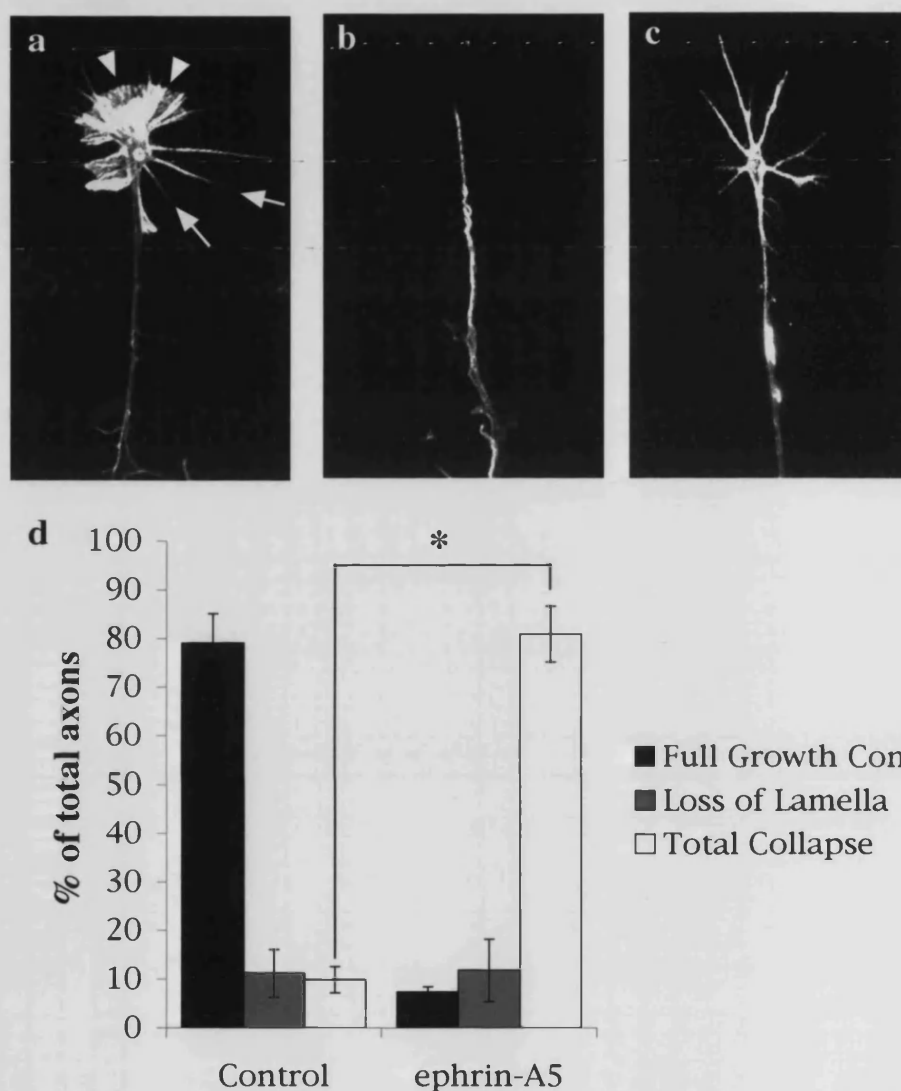
($p > 0.9$, Fisher's exact test for independence). Similarly mock injection has no effect on the percentage of RGC axons that retract in response to contact with an MDCK cell (see Table 3.1, $p > 0.3$, Fisher's exact test for independence). Taken together these data suggest that expression of ephrin-A5 on the surface on an MDCK is sufficient to induce a repulsive RGC response similar to that induced by contact with a fibroblast that endogenously expresses ephrin-As.

3.2.7. Soluble ephrin-A5-Fc causes loss of RGC lamellae and axon retraction.

Many published studies have reported that ephrin-A5, presented in soluble form, triggers collapse of RGC growth cones, defined as the complete loss of the F-actin-rich lamella and filopodia that comprise the axonal growth cone (Cheng et al., 2003; Drescher et al., 1995; Monschau et al., 1997; Wahl et al., 2000; Weinl et al., 2003; Wong et al., 2004). Few of these reports describe the response of RGCs dynamically, and none address whether the loss of RGC lamella can occur separately from the loss of filopodia and axon retraction, as is seen in response to an endogenous ephrin-A-expressing cell. To extend the published results I have studied the response of RGCs to soluble ephrin-A5 in detail and dynamically, in particular to investigate the possibility that soluble ephrin-A5 triggers a series of temporally distinct events. Addition of ephrin-A5-Fc to the bathing medium of nasal RGCs in culture will also allow comparison of the RGC responses induced by soluble ephrin and those induced by contact with an ephrin-A-expressing cell.

EphA4, expressed on nasal RGC axons in culture, is required for the repulsive response of these axons to substrate-bound ephrin-A5 (Monschau et al., 1997; Walkenhorst et al., 2000). Ephrin-A5-Fc dimers fail to induce phosphorylation of EphA4 unless they are clustered in to higher order oligomers, and similarly ephrin-A5-Fc only inhibits outgrowth of EphA4-positive motor neurons when it is clustered (Ohta et al., 1997). Ephrin-A5-Fc was therefore clustered with an anti-human antibody (see section 2.7.2) before it was added to the bathing medium of nasal RGCs in culture at a final concentration of $1 \mu\text{gml}^{-1}$. Nasal RGCs in culture have a characteristic growth cone that consists of F-actin-rich filopodia (Fig 3.10a arrows) and a veil-like lamella (Fig 3.10a arrowheads). Stimulation of RGCs with clustered ephrin-A5-Fc for 10 min results in the complete loss of the growth cone lamella and

Figure 3.10. Soluble ephrin-A5-Fc induces loss of RGC growth cone lamella and filopodia.

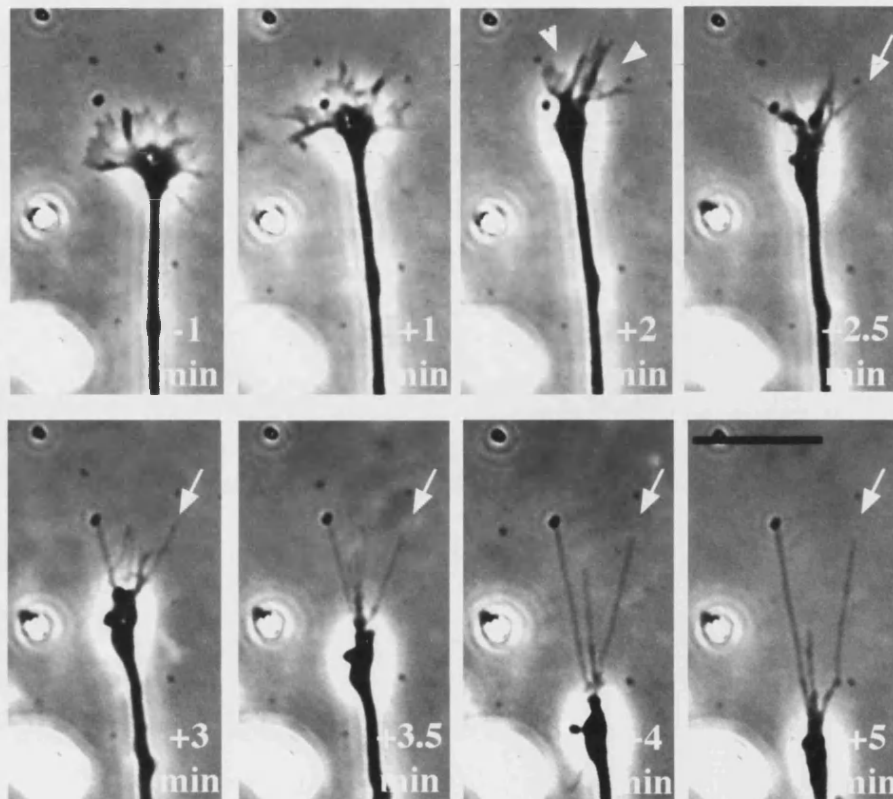


Phalloidin staining shows that RGCs in culture, treated with anti-Fc as a control (a) have full growth cones with spread lamella (arrowheads) and filopodia (arrows). Stimulation with clustered ephrin-A5-Fc (1 µg/ml) for 10 minutes triggers loss of growth cone lamella and filopodia (b). In order to quantify the response to soluble ephrin-A5-Fc, retinal explants were stimulated with clustered ephrin-A5-Fc (1 µg/ml) for 10 min before being fixed, stained with phalloidin and categorised as having a full growth cone (for example see a), having completely lost lamella but retaining more than 3 filopodia ("Loss of lamella", for example see c), or having lost lamella and having 3 or fewer filopodia/retraction fibres ("Total collapse", for example see b). Quantification of the RGC response to soluble ephrin-A5 demonstrates that the percentage of RGCs that exhibit total collapse in response to stimulation with ephrin-A5-Fc (1 µg/ml) for 10 minutes is significantly higher than the percentage of totally collapsed control RGCs (d, * $p < 0.001$, student's T-test for 2-tailed data of unequal variance).

filopodia (Fig 3.10b). In order to quantify the response of a large number of RGC axons to soluble ephrin-A5-Fc, retinal explants were fixed after 10 min stimulation with $1\mu\text{gml}^{-1}$ ephrin-A5-Fc, and stained with fluorescently labelled phalloidin in order to visualise the morphology of their actin cytoskeleton. RGCs classified as having a “full growth cone” show spread lamella and filopodia (for example Fig 3.10a). In order to investigate whether stimulation with soluble ephrin-A5 can trigger loss of growth cone lamellæ without subsequent loss of filopodia, as can occur in response to contact with an ephrin-A-expressing cell, RGC axons meeting the strict criteria of complete loss of lamellæ but having more than 3 filopodia are categorised as “loss of lamella” (for example Fig 3.10c). The final category, “total collapse”, is defined as complete loss of lamellæ with 3 or fewer F-actin positive filopodia or retraction fibres at the end of the axon (for example Fig 3.10b). In the control situation the majority ($79.0 \pm 6.0\%$) of RGC axons have a full growth cone, but stimulation with ephrin-A5-Fc induces loss of both lamellæ and filopodia to cause total collapse in $80.9 \pm 5.7\%$ of the axons (Fig 3.10d). The percentage of RGC axons that exhibit total collapse in response to ephrin-A5-Fc is significantly higher than the percentage of control RGCs in this category ($9.8\% \pm 2.7\%$, $p < 0.001$ Student’s T-Test for 2-tailed data of unequal variance). There is a small percentage ($11.8 \pm 6.4\%$) of RGCs that lose only lamellæ in response to stimulation with ephrin-A5-Fc (Fig 3.10d), but this is not significantly higher than the percentage of control RGCs with this morphology ($11.1 \pm 4.9\%$, $p > 0.8$ Student’s T-Test for 2-tailed data of unequal variance). This suggests that, unlike contact with an endogenous ephrin-A-expressing cell (see section 3.2.2), stimulation with soluble ephrin-A5-Fc does not cause loss of lamella independent of loss of filopodia.

Analysis of individual RGCs by time-lapse microscopy reveals that the response of a RGC to ephrin-A5-Fc is extremely rapid (Fig 3.11). The RGC growth cone lamella has already begun to collapse 2 min after addition of ephrin-A5-Fc (Fig 3.11 arrowheads) and is completely lost by 2.5 min. After the lamella is completely lost the axon begins to retract (Fig 3.11). On average the lamella of an ephrin-A5 stimulated RGC is completely lost by 2.47 ± 0.49 min ($n=6$). The axon retraction is slightly delayed, beginning on average 2.58 ± 0.57 min after ephrin addition. Examination of each individual RGC reveals that in the majority (83.3%) of cases the lamella has been lost completely before the axon begins to retract ($n=6$),

Figure 3.11. Soluble ephrin-A5-Fc induces rapid loss of RGC growth cone lamella, followed by axon retraction.



Time-lapse stills showing that stimulation with clustered ephrin-A5-Fc (1 μ g/ml) induces rapid loss of RGC growth cone lamella and filopodia, and axon retraction. Within 2 min of ephrin-A5 addition the growth cone lamella begins to collapse (arrowheads), and by 2.5 min the lamella is completely lost. After the RGC growth cone lamella is completely lost the axon begins to retract, and as it does most of the growth cone filopodia are dragged away, leaving behind only a few retraction fibres, which were initially filopodia (arrows). Scale bar = 20 μ m

similar to the sequential response seen in RGC growth cones following contact with a Swiss-3T3 fibroblast.

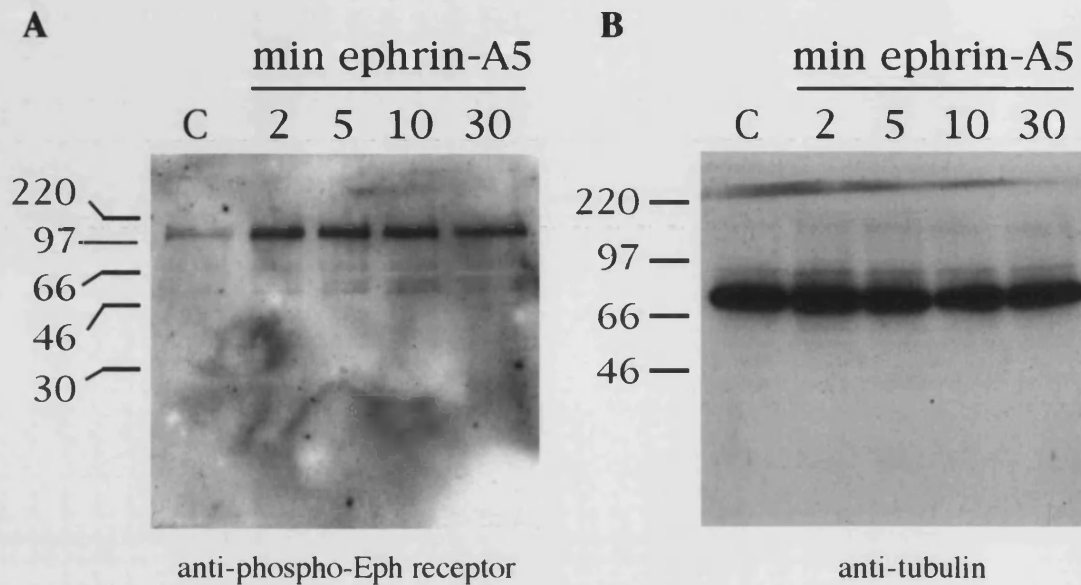
It is more difficult to quantify the timing of filopodia loss in response to soluble ephrin-A5. Filopodia are largely hidden by the lamella of a growth cone prior to ephrin-A5-Fc addition (Fig 3.11) making it impossible to compare filopodial behaviour before and after ephrin stimulation. It is clear however that most of the RGC growth cone filopodia are dragged away as the axon retracts (Fig 3.11, Movie 3.11). Similar to contact with an ephrin-A-expressing cell, a few retraction fibres, which appear to be filopodial in origin, remain as the axon retracts (Fig 3.11, arrows). Axon retraction in response to soluble ephrin-A5-Fc occurs in all cases recorded by phase time-lapse microscopy (n=6), and in none of these cases do more than three retraction fibres remain after 90s of axon retraction. Although the strict criteria for assessing total collapse in fixed cultures does not formally address whether these axons have retracted, the above observations suggest that ephrin-A5-Fc-treated RGC axons that have 3 or fewer F-actin positive filopodia/retraction fibres (meeting the criteria for total collapse) are likely to have retracted.

In order to correlate the repulsive RGC response to ephrin-A5-Fc with neuronal Eph receptor activation, I have blotted RGC lysates with the antibody raised against Eph receptors phosphorylated at the juxtamembrane tyrosine residues. This antibody detects a major band at around 110kD, which correspond to full-length phosphorylated Eph receptors (Becker et al., 1995), as well as minor bands at around 50kD (Fig 3.12A, see also Fig 4.10). These lower bands are likely to be degradation products, as they are induced with the same time course as the major band (Fig 3.12). Stimulation of isolated RGCs in culture with clustered ephrin-A5-Fc induces a rapid and sustained increase in Eph receptor phosphorylation (Fig 3.12A). RGC Eph receptors are substantially activated after 2 min of ephrin-A stimulation, at which time RGCs begin to lose their growth cone lamella (Fig 3.11). RGC lysates were also blotted with an anti-tubulin antibody to control for protein levels (Fig 3.12B).

3.3. Discussion.

In Chapter 3 I have described a robust and reproducible assay with which to investigate the response of an individual nasal RGC growth cone to contact with a single Swiss-3T3 fibroblast that expresses endogenous ephrin-A. The RGC response

Figure 3.12. Stimulation of isolated RGCs with ephrin-A5-Fc induces rapid and sustained activation of Eph receptors.



Isolated RGCs in culture were treated with anti-Fc as a control (C) or stimulated with clustered ephrin-A5-Fc (1 μ g/ml) for the time indicated. Cells were lysed with RIPA buffer and lysates were separated by SDS-PAGE and blotted with anti-phospho-Eph receptor antibody (A) to monitor receptor activation. Stimulation with ephrin-A5 results in rapid and sustained increase in Eph receptor phosphorylation. Cell lysates were blotted with anti-tubulin antibody (B) as a control for protein levels.

It should be noted, as mentioned in section 2.4, the anti-phospho-Eph receptor antibody used was not affinity purified against the phosphopeptide, and therefore the bands shown here may also reflect increased phosphorylation of other proteins.

to contact with a Swiss-3T3 fibroblast consists of rapid loss of RGC growth cone lamella, which is often followed by axon retraction. Contact with a Swiss-3T3 fibroblast induces activation of Eph receptors on the RGC growth cone, and disruption of the EphA-ephrin-A interaction prevents the contact-induced loss of RGC growth cone lamellæ and axon retraction. Contact with a cell type that does not express ephrin-As results in a much-reduced incidence of lamellæ loss and axon retraction, and overexpression of ephrin-A5 in this cell type is sufficient to induce the repulsive response. Together the data presented in this chapter demonstrate that RGC EphA signalling mediates a series of distinct cellular events induced by contact with a Swiss-3T3 fibroblast. The assay described in this chapter allows detailed analysis of nasal RGC behaviour in response to an individual endogenous ephrin-A-expressing cell, which is physiologically relevant since ephrin-As are membrane tethered *in vivo*. Therefore this novel assay, used in conjunction with the traditional soluble collapse assay, provides a powerful tool with which to identify signalling pathways that mediate the distinct events that comprise the repulsive response; loss of RGC lamella, and subsequent loss of filopodia and axon retraction.

3.3.1. Comparison with previous studies of contact-mediated RGC collapse.

The repulsive response of a nasal RGC to contact with a Swiss-3T3 fibroblast consists of a series of temporally distinct events. The RGC growth cone lamella rapidly collapses, and in many cases this is followed by axon retraction concomitant with loss of filopodia. Analysis of contact-induced RGC responses in this detail has not been described previously, as published studies do not separate these events (Davenport et al., 1996; Davenport et al., 1998; Davenport et al., 1999; Thies and Davenport, 2003). The nasal RGC response to contact with a Swiss-3T3 fibroblast described in this chapter can be compared to previous reports from Roger Davenport and colleagues describing the behaviour of temporal RGCs following contact with isolated tectal cells (Davenport et al., 1996; Davenport et al., 1998; Davenport et al., 1999; Thies and Davenport, 2003). In these studies the authors report that contact with isolated chick tectal neurons induces around 80% of temporal RGC axons to retract while nasal RGCs are unresponsive (Davenport et al., 1996; Davenport et al., 1999; Thies and Davenport, 2003). In this chapter I demonstrate that 43.3% of nasal

RGC axons retract in response to contact with a Swiss-3T3 fibroblast. Temporal RGCs in culture express higher levels of EphA3 than nasal axons, and are more sensitive to ephrin-A2 and ephrin-A5, both of which are expressed by the tectal neurons that induce temporal RGC axon retraction (Davenport et al., 1996; Davenport et al., 1998; Monschau et al., 1997; Nakamoto et al., 1996). I could not determine which members of the ephrin-A family Swiss-3T3 fibroblasts express under co-culture conditions. It is possible that a combination of differential EphA receptor expression on nasal and temporal RGC axons, and a different complement of ephrin-As expressed on the contacting cell accounts for the differences in the frequency of axon retraction reported in this chapter and the published studies. However, it is important to note that the authors of the published studies have not addressed whether the repulsive RGC response they describe is mediated by Eph-ephrin interaction.

The repulsive axonal guidance cue RGM (repulsive guidance molecule), which is unrelated to the ephrins, is expressed in an increasing anterior to posterior gradient in the chick tectum (Monnier et al., 2002). RGM is expressed on isolated chick tectal neurons *in vitro* [B.K. Muller, unpublished observations reported in (Muller et al., 1996)], and temporal but not nasal RGC axons *in vitro* are repelled by both soluble and substrate-bound RGM (Monnier et al., 2002; Muller et al., 1996). The repulsive response of temporal RGCs to contact with chick tectal neurons, described by Roger Davenport and colleagues, may therefore be the result of signalling pathways induced by RGM, instead of, or in addition to, ephrin-As. In this chapter I have demonstrated that addition of EphA5-Fc to the bathing medium of RGCs and Swiss-3T3 fibroblasts in co-culture, which will disrupt EphA-ephrin-A interaction at sites of cell-cell contact (Marcus et al., 2000), completely abolishes nasal RGC axon retraction in response to contact with a fibroblast. Together with the observation that MDCK cells induce very little axon retraction unless they over-express ephrin-A5, this provides strong evidence that nasal RGC axon retraction in response to Swiss-3T3 fibroblast, although occurring at a lower frequency than the temporal axon retraction induced by contact with a tectal neuron, is entirely mediated by this EphA-ephrin-A interaction. Given that contact-induced loss of RGC lamellæ is similarly dependent on EphA-ephrin-A interaction, the RGC repulsive response to contact with a Swiss-3T3 fibroblast described in this chapter provides a robust, reproducible

and physiologically relevant assay with which to study the signalling pathways that mediate the neuronal responses to ephrin-A stimulation.

3.3.2. The contact-induced responses in the ephrin-A expressing cell are not a result of EphA or ephrin-A signalling

Approximately half of RGC-Swiss-3T3 interactions result in withdrawal of the fibroblast at the site of cell-cell contact. There are several reasons why it is unlikely that reverse signalling through ephrin-A ligands expressed on the surface of the Swiss-3T3 fibroblast mediates this withdrawal response. Withdrawal of MDCK cells, which do not express ephrin-As, also occurs in around 50% of interactions with RGCs, and expression of ephrin-A5 does not increase the incidence of MDCK cell withdrawal, in fact it reduces the MDCK response (Table 3.2). Activation of ephrin-A signalling in fibroblasts promotes cellular protrusion (Davy and Robbins, 2000), suggesting that activation of ephrin-A-mediated signalling following contact with a RGC would not result in withdrawal of the contacting cell. RGCs in culture express ephrin-As (Hornberger et al., 1999) and both Swiss-3T3 fibroblasts (C.D. Nobes unpublished data) and MDCK cells (Miao et al., 2003) in culture express endogenous EphA receptors, but addition of EphA5-Fc to the bathing medium does not significantly reduce the percentage of Swiss-3T3 fibroblasts that withdraw after contact with a RGC (Table 3.2). This suggests that neither ephrin-A- nor EphA-mediated signalling is responsible for the non-neuronal cell response, and therefore this response is not a suitable assay with which to investigate these signalling pathways.

3.3.3. Comparison between the RGC response to soluble and membrane-tethered ephrin-A.

Ephrin-As are membrane-tethered molecules *in vivo* (Gale et al., 1996b; Pandey et al, 1995a), and therefore the co-culture assay described in this chapter is a more physiologically relevant assay with which to study the RGC response to stimulation with ephrin-A than the traditional soluble collapse assay. However, the soluble collapse assay has an advantage in that it can be used to check for cell autonomy in the effect of pharmacological inhibitors. Overall the RGC response to soluble ephrin-A5-Fc is very similar to that induced by contact with a Swiss-3T3 fibroblast, and

therefore the soluble collapse assay can be used in combination with the novel co-culture assay described in this chapter to identify signalling molecules that mediate the various aspects of the RGC response to ephrin-A. The only significant difference between the RGC response to soluble ephrin-A5-Fc and contact with a Swiss-3T3 fibroblast is that stimulation with soluble ephrin-A5-Fc induces loss of RGC growth cone lamellæ and axon retraction more rapidly. It is possible that this difference reflects a variation in the ligands that induce the repulsive response, because I could not determine which members of the ephrin-A family Swiss-3T3 fibroblasts express under co-culture conditions. Of the ephrin-A ligands tested to date however, only ephrin-A5 induces significant growth cone collapse of nasal RGCs *in vitro* (Menzel et al., 2001; Monschau et al., 1997), which might suggest that the complement of ephrin-As expressed by Swiss-3T3 fibroblasts in culture includes ephrin-A5.

It is possible that $1\mu\text{gml}^{-1}$ soluble ephrin-A5 is a higher effective concentration of ligand than that presented on the surface of a cell that expresses endogenous levels of ephrin-A, such as a Swiss-3T3 fibroblast. Chick RGCs *in vivo* express multiple EphA receptors, and to date expression of three of these has been demonstrated *in vitro* (Connor et al., 1998; Holash and Pasquale, 1995; Monschau et al., 1997). It is possible that a high concentration of ephrin-A5 may activate additional, lower affinity EphA receptor to those activated by endogenous levels of ephrin-A presented on the surface of a Swiss-3T3 fibroblast, and thus cause an amplified and more rapid response. However, MDCK-ephrin-A5 cells express much higher levels of ephrin-A expression than Swiss-3T3 fibroblasts, since surface ephrin-A5 is easily detected using an anti-ephrin-A5 antibody (Fig 3.9C). Contact with an MDCK-ephrin-A5 cell does not result in a significantly faster lamellæ collapse ($p>0.3$) or axon retraction ($p>0.6$) compared to contact with a Swiss-3T3 fibroblast, which suggests that differences in the effective ephrin-A concentration does not account for the difference in the latency of RGC response to soluble and membrane bound ephrin-A.

Stimulation of RGCs with ephrin-A2 and ephrin-A5 leads to increased fluid phase endocytosis with a time-course that matches ephrin-induced growth cone collapse (Fournier et al., 2000; Journey et al., 2002). Blocking the activity of Rac, which prevents this ephrin-induced endocytosis, also inhibits ephrin-induced growth cone collapse, and the authors suggest that endocytosis is required for loss of growth cone structures during the neuronal response to repulsive axon guidance cues (Journey et

al., 2002). It has also been shown that following contact with an EphA-expressing axon ephrin-A2 is proteolytically cleaved from the surface of a transfected cell, and is internalised by the axon during subsequent axon retraction (Hattori et al., 2000). Cleavage of membrane-bound ephrin-A2 commences 10 min after EphA binding, and, although it must be noted that uptake of soluble ephrin-A was not measured in the published studies, fluid phase endocytosis was stimulated within 5 min of soluble ephrin-A addition (Hattori et al., 2000; Journey et al., 2002). If endocytosis of ephrin-A is necessary to induce the loss of RGC growth cone lamella and axon retraction described in this chapter, the more rapid response to soluble versus membrane bound ephrin-A may reflect the fact that soluble ephrin-A can be internalised more quickly than membrane bound ephrin-A, which may need to be cleaved first. Soluble ephrin-A5 added to the bathing medium has the potential to simultaneously bind EphA receptors all over the growth cone, as well as available EphA receptors along the RGC axon shaft. Global EphA activation and/or ephrin-A internalisation at multiple sites simultaneously may contribute to the more rapid repulsive response to soluble ephrin-A-5.

The slightly slower response of RGCs to contact with a Swiss-3T3 fibroblast reveals distinct cellular events that are difficult to separate in response to soluble ephrin-A. Since ephrin-As are membrane bound *in vivo*, the cellular responses to endogenous ephrin-A on the surface of a Swiss-3T3 fibroblast are likely to reflect the physiological consequences of neuronal EphA receptor activation. In support of this idea, *in vivo* analysis of RGC behaviour at the optic chiasm, where repulsion by ephrin-As is thought to contribute to RGC axon guidance, reveals dynamic extension and retraction of filopodia and lamella, and periods of axon extension and retraction (Godement et al., 1994; Marcus et al., 2000). The novel RGC-Swiss-3T3 co-culture assay described in this chapter allows quantification of the various aspects of the RGC response to stimulation with ephrin-A and, used in conjunction with the soluble collapse assay, will provide a powerful tool with which to investigate the signalling pathways that mediate loss of growth cone lamellæ and axon retraction during the RGC response to stimulation with ephrin-A.

Chapter 4. The roles of ROCK and Src family kinases in ephrin-A-induced RGC repulsive responses.

4.1. Introduction.

Numerous signalling proteins and adaptor molecules that interact with Eph receptors in an activity-dependent manner have been identified (see section 1.5), but the signal transduction pathways downstream of Eph receptors remain far from fully characterised. In an effort to identify signals involved in the repulsive RGC response to membrane-bound ephrin-A, I have concentrated on two potential signalling intermediates previously implicated in axon guidance and Eph receptor signalling, the RhoA-associated kinase, ROCK, and members of the Src family of non receptor tyrosine kinases.

Rho family GTPases are major regulators of the actin cytoskeleton, and have important roles in axon guidance (Dickson, 2002). In particular Rho activity has been linked to repulsive axon guidance mediated by ephrin-A, since the level of active Rho in RGCs increases following stimulation with soluble ephrin-A5 (Wahl et al., 2000). One possible mechanism for this increase is via the guanine exchange factor (GEF) Ephexin, which binds to EphA4 (Shamah et al., 2001). *In vitro* Ephexin catalyses RhoA guanine nucleotide exchange, and a dominant negative form of Ephexin, which cannot increase Rho activity, inhibits ephrin-A-induced collapse of rat RGC growth cones (Shamah et al., 2001).

ROCK, an effector of Rho, directly phosphorylates the regulatory light chain of myosin II (myosin light chain, MLC), and this leads to the assembly of contractile actin-myosin filaments (Amano et al., 1996; Citi and Kendrick-Jones, 1987). Expression of a constitutively active mutant of ROCK is sufficient to induce neurite retraction (Amano et al., 1998) and inhibiting the activity of Rho or ROCK in chick RGCs, with C3 toxin or the pharmacological inhibitor Y27632 respectively, has been shown to inhibit the growth cone collapse induced by soluble ephrin-A5 (Cheng et

al., 2003; Wahl et al., 2000). Interestingly, expression of dominant negative Ephexin inhibits ephrin-A-induced growth cone collapse less than inhibition of Rho and Rock (Shamah et al., 2001; Wahl et al., 2000), suggesting that an additional mechanism may modulate the Rho-ROCK pathway in RGCs in response to ephrin-A.

Genetic manipulation in *Drosophila* has provided evidence that the Rho-ROCK-MLC pathway is involved in axon retraction *in vivo*, and that this pathway is negatively regulated by p190RhoGAP (Billuart et al., 2001). Interestingly reducing Src expression suppresses the axon retraction phenotype seen in p190RhoGAP mutants, suggesting that Src activity may positively regulate Rho dependent axon retraction *in vivo* (Billuart et al., 2001).

Src is enriched in the axon tracts and growth cone membrane fractions of RGCs *in vivo*, and both Src and Fyn are expressed in growth cones of chick RGCs in culture (Burden-Gulley and Lemmon, 1996; Maness et al., 1988; Sorge et al., 1984). EphA4, which is expressed by chick RGCs *in vitro* and is required for the repulsive response of nasal RGC axons to ephrin-A5 (Monschau et al., 1997; Walkenhorst et al., 2000), interacts with Fyn *in vitro* and in whole cells (Ellis et al., 1996; Prevost et al., 2002). Ephrin-stimulation of a variety of cell types, including primary neurons, in culture results in an increase in Src and Fyn activity (Sharfe et al., 2003; Steinle et al., 2002; Takasu et al., 2002; Vindis et al., 2003) and Src kinases have been functionally linked to Eph receptor signalling, since pharmacological inhibition of Src kinase activity inhibits ephrin-induced migration of endothelial cells (Steinle et al., 2002; Vindis et al., 2003).

RGC growth cone collapse in response to tectal membranes involves co-ordinated regulation of the actin cytoskeleton and cell-matrix adhesions (Cox et al., 1990), and recently it has been shown that ECM molecules can influence the RGC response to ephrin-A5 (Weinl et al., 2003). Since Src family kinases are known to play a role in the turnover of focal adhesions and mediating integrin signals (Carragher et al., 2001; Cary et al., 1999; Fincham et al., 1995; Fincham and Frame, 1998; Webb et al., 2004), they are good candidates for mediating or regulating the repulsive RGC response to ephrin-A stimulation.

In an effort to characterise the signalling pathways downstream of EphA receptors I have used specific inhibitors of ROCK (Y27632) and Src family kinases (PP2), to

investigate the role of these signalling molecules in the ephrin-induced RGC repulsive response. I have determined the effect of these inhibitors on the behaviour of RGC axons following contact with an ephrin-A-expressing Swiss 3T3 fibroblast, and after stimulation with soluble ephrin-A5, and demonstrate that while ROCK is involved in ephrin-A-induced axon retraction, it does not mediate ephrin-A-induced loss of lamella. By contrast it appears that Src family kinase activity is not required for ephrin-A-induced growth cone collapse and axon retraction.

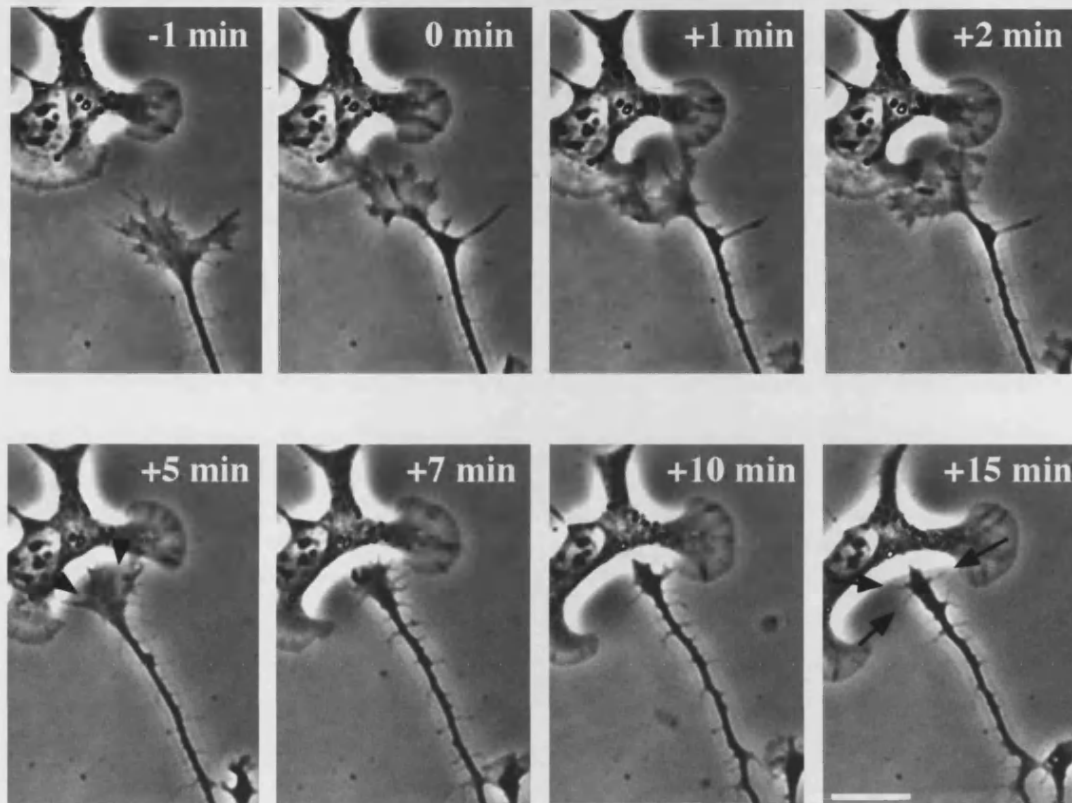
4.2. Results.

4.2.1. The ROCK inhibitor Y27632 prevents RGC axon retraction, but not loss of lamellae in response to contact with an ephrin-A-expressing cell.

To determine the role of ROCK activity in the repulsive response of RGCs to stimulation with membrane-bound ephrin-As I have investigated the effect of Y27632 on the behaviour of RGCs in response to contact with Swiss 3T3 fibroblasts, which endogenously express ephrin-As (see Chapter 3). Y27632 is a specific inhibitor of ROCK activity, with a similar IC_{50} for both ROCK isoforms (Ishizaki et al., 2000; Uehata et al., 1997). Treatment of RGCs with 10 μ M Y27632 for 1 hour results in complete inhibition of ROCK activity in these cells, assayed by phosphorylation levels of MLC (Wahl et al., 2000). Co-cultures of retinal explants and Swiss 3T3 fibroblasts were therefore incubated with 10 μ M Y27632 for 1-2 hours prior to cell-cell contact in order to address the role of ROCK during the repulsive RGC response to ephrin-A.

An example of the interaction between a RGC growth cone and an ephrin-A-expressing fibroblast following incubation with Y27632 is shown in Fig 4.1 and Movie 4.1. Following contact with the fibroblast the RGC growth cone continues to advance for several minutes before the lamella begins to retract (Fig 4.1 arrowheads). The growth cone lamella is completely lost by 10 min post-contact, and following loss of lamella the axon ceases to advance (Fig 4.1). Inhibition of ROCK does not inhibit the loss of RGC growth cone lamellæ in response to contact with an ephrin-A-

Figure 4.1 The ROCK inhibitor Y27632 prevents RGC axon retraction but not loss of growth cone lamella in response to contact with an ephrin-A-expressing fibroblast.



Time-lapse stills showing that Y27632 prevents RGC axon retraction, but not loss of lamella, in response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast. Y27632 (10 μ M) was added 1-2 hours prior to cell contact and times shown are relative to initial contact. Following contact with the fibroblast, the RGC growth cone continues to advance a short distance before the lamella begins to collapse at around 5 minutes post-contact (see arrowheads). The RGC growth cone lamella is not completely lost until 10 minutes after contact, significantly delayed compared to control (see text). The RGC axon does not retract within 15 minutes of contact, and retains filopodia over this period (see arrows). Scale bar = 20 μ m.

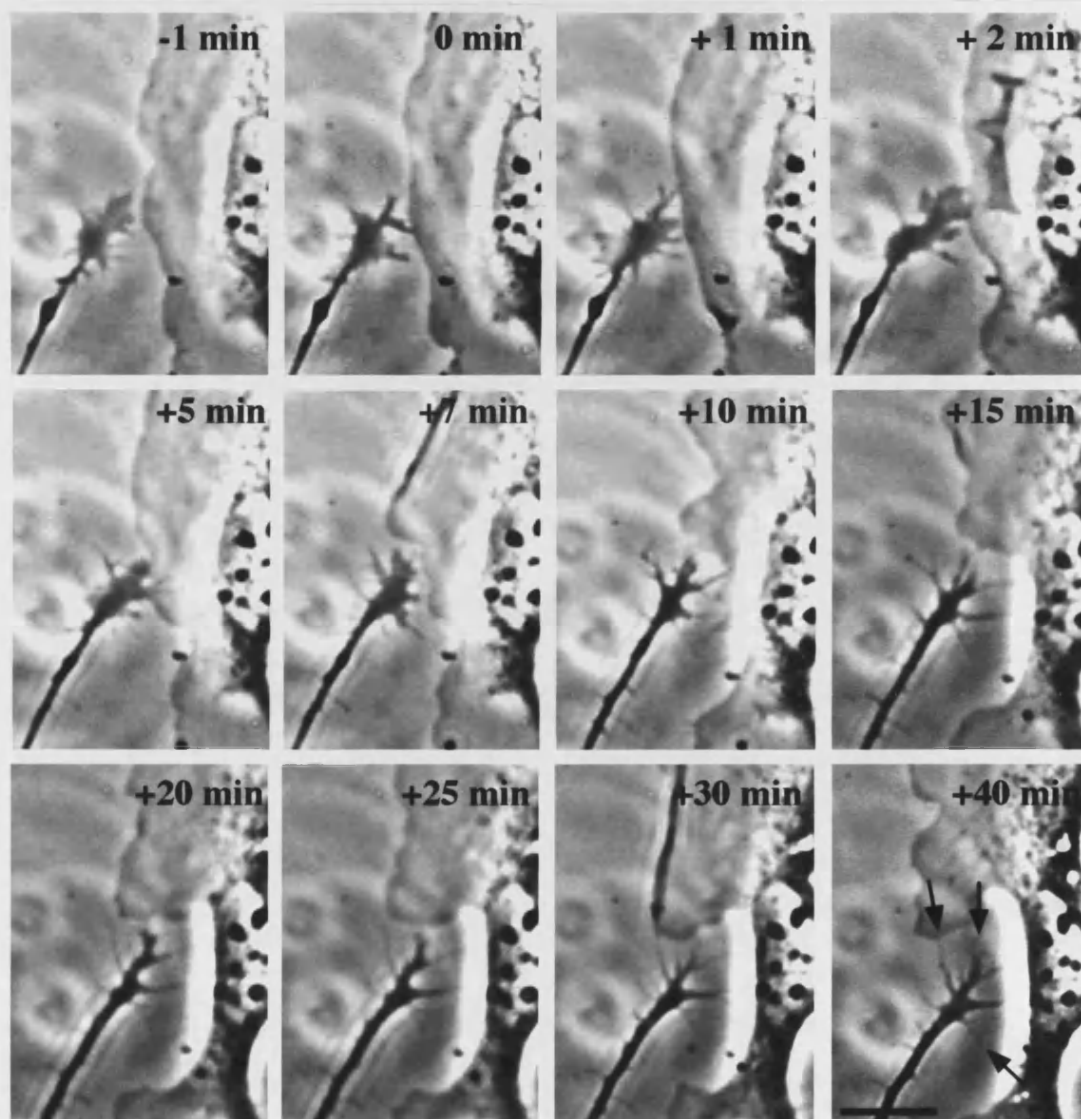
Table 4.1

Cell type and treatment (n)	RGCs showing loss of lamella within 20 min of contact.	RGCs showing axon retraction within 20 min of contact.
Swiss 3T3 fibroblast (30)	83.3%	43.3%
Swiss 3T3 fibroblast + Y27632 (15)	93.3%	6.7%

expressing fibroblast; 93.3% of Y27632-treated RGCs lose lamellæ within 20 min of contact with a Swiss-3T3 fibroblast, compared with 83.3% of control, untreated axons (see Table 4.1, $p > 0.8$; Fisher's exact test for independence). Treatment with Y27632 does however delay the RGC loss of lamellæ in response to contact with an ephrin-A-expressing fibroblast. On average complete loss of Y27632-treated RGC lamellæ occurs at 11.2 ± 4.6 min ($n=15$) compared with 5.9 ± 2.6 min for control RGCs ($n=25$, $p < 0.001$; Student's T-Test for 2-tailed data of unequal variance).

The Y27632-treated RGC axon shown in Fig 4.1 retains filopodia following lamella loss (Fig 4.1 arrows). As discussed in Chapter 3 the presence of filopodia following ephrin-A stimulation correlates with lack of axon retraction, and Fig 4.1 demonstrates that Y27632 prevents RGC axon retraction within 15 min of contact with an ephrin-A-expressing fibroblast. Quantification of 15 RGC-fibroblast interactions in the presence of Y27632 reveals that only 6.7% of Y27632-treated axons retract within 20 min of contact with an ephrin-A-expressing fibroblast, compared with 43.3% of control axons (see Table 4.1, $p < 0.02$; Fisher's exact test for independence). Since the presence of Y27632 significantly delays ephrin-A-induced loss of RGC lamella, it is possible that axon retraction is similarly delayed. Not all RGC-fibroblast interactions in the presence of Y27632 were recorded for longer than 20 min, but the interaction shown in Fig 4.2 is an example filmed over a greater period of time. Although the RGC lamella is completely lost 10 min after contact the axon does not retract up to 40 min after contact with the ephrin-A-expressing fibroblast, and retains filopodia over this time course (Fig 4.2 arrows). Of the 9 Y27632-treated RGC-fibroblast interactions recorded beyond 20 min after initial

Figure 4.2 The Y27632-dependent inhibition of axon retraction is not the result of a delayed RGC response to contact with an ephrin-A-expressing fibroblast.



Time-lapse stills showing that RGC axon retraction is not delayed following contact with an ephrin-expressing Swiss-3T3 fibroblast. Y27632 (10 μ M) was added 1-2 hours prior to cell contact and times shown are relative to initial contact. RGC lamella loss is not complete until 10 minutes after contact, but the RGC axon does not retract within 40 minutes, and the RGC retains filopodia throughout the period of contact with the fibroblast cell (see arrows). Scale bar = 20 μ m.

contact, 8 do not result in axon retraction within the duration of recording (30-45 min post contact), although all lose lamella within 12 min. Together these data demonstrate that inhibition of ROCK prevents axon retraction, and delays but does not prevent loss of RGC lamella in response to stimulation with membrane-tethered ephrin-A.

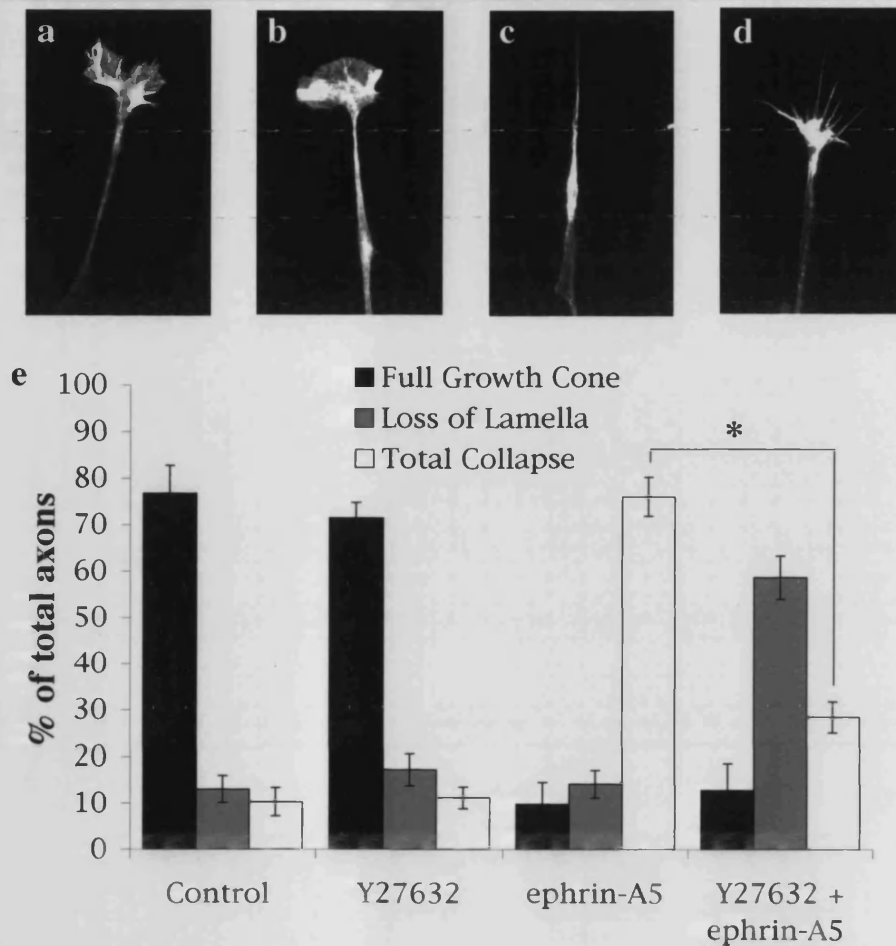
4.2.2. The ROCK inhibitor Y27632 prevents ephrin-A5-Fc-induced loss of RGC filopodia and axon retraction.

It has previously been reported that Y27632 significantly reduces the percentage of RGC axons that lose both lamellæ and filopodia in response to soluble ephrin-A5 (Cheng et al., 2003; Wahl et al., 2000). As described above, Y27632 specifically inhibits loss of filopodia, but not lamellæ in response to membrane tethered ephrin-A. In order to determine whether this effect of Y27632 is conserved in the response of RGC growth cones to stimulation with soluble ephrin-A5, and to confirm that the effects of Y27632 are RGC-autonomous, I have investigated the effect of Y27632 on the loss of RGC lamella or filopodia, or both, following addition of ephrin-A5-Fc.

Typical examples of the actin morphology of RGC growth cones treated with ephrin-A5-Fc and/or Y27632 are shown in Fig 4.3. Incubation with 10 μ M Y27632 (Fig 4.3b) has no effect on the actin morphology of RGC growth cones compared to control (Fig 4.3a); both have spread lamellæ and filopodia. Stimulation of RGCs with 1 μ gml⁻¹ ephrin-A5-Fc for 10 min induces the loss of growth cone lamellæ and filopodia (Fig 4.3c). Although the ephrin-A5-induced loss of lamella is not inhibited by Y27632 treatment, the growth cone retains filopodia (Fig 4.3d), and therefore, as discussed in Chapter 3, is unlikely to have retracted.

Quantification of the effect of ROCK inhibition on the response of RGCs to stimulation with soluble ephrin-A5 is shown in Fig 4.3e. Incubation with Y27632 does not affect growth cone morphology, since the percentage of axons with a full growth cone is not significantly changed ($71.5 \pm 3.4\%$ of Y27632-treated RGCs compared with $76.8 \pm 6.0\%$ of control, $p > 0.1$; Student's T-Test for 2-tailed data of unequal variance). As shown in Chapter 3, stimulation with ephrin-A5-Fc (1 μ gml⁻¹) for 10 min induces total collapse (loss of growth cone lamellæ and filopodia) in the majority ($76.1 \pm 4.2\%$) of RGC axons (Fig 4.3e). Pre-treatment of RGCs with Y27632 (10 μ M) for 20 min significantly rescues the percentage of axons that exhibit

Figure 4.3 The ROCK inhibitor Y27632 does not prevent the loss of RGC growth cone lamellae in response to soluble ephrin-A5, but does prevent loss of growth cone filopodia.



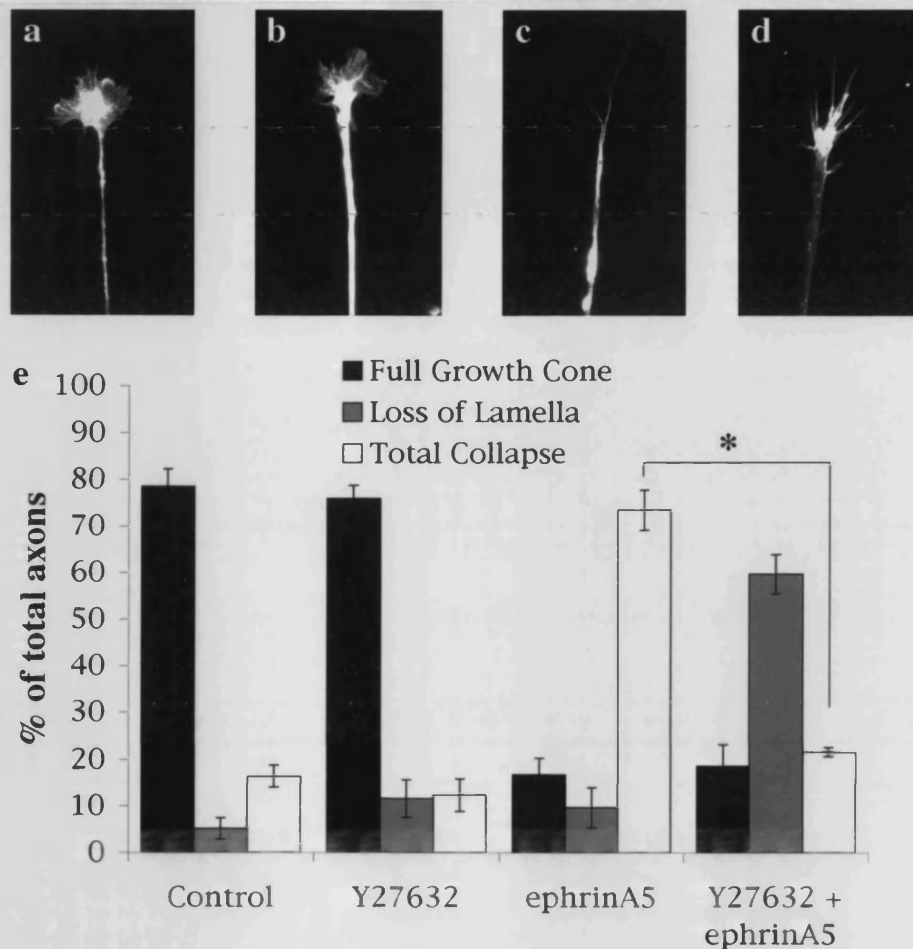
Y27632 inhibits ephrin-A5-Fc-induced loss of RGC filopodia but not loss of lamella. Phalloidin staining shows that RGCs treated with anti-Fc control have full growth cones with spread lamella and filopodia (a). Treatment with Y27632 (10 μ M) alone for 1 hour has no effect on the f-actin morphology of growth cones (b). Stimulation with clustered ephrin-A5-Fc (1 μ g/ml) for 10 minutes triggers loss of growth cone lamella and filopodia (c). Y27632 prevents the loss of filopodia in response to ephrin-A5, but does not prevent the loss of lamella (d). Quantification of the effect of Y27632 on ephrin-A5-treated RGCs demonstrates that pre-treatment with Y27632 (10 μ M 1 hour) significantly reduces the percentage of RGCs that lose both filopodia and lamella (Total collapse) in response to stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes (e, * $p < 0.0001$, student's T-test for 2-tailed data of unequal variance). The majority ($58.7 \pm 4.6\%$) of Y27632-treated RGCs lose lamella but not filopodia following stimulation with-A5.

total collapse ($28.5 \pm 3.4\%$, $p < 0.0001$; Student's T-Test for 2-tailed data of unequal variance, see Fig 4.3e). Instead of having a full growth cone however, the majority ($58.7 \pm 4.7\%$) of Y27632-treated RGCs lose lamellæ but retain filopodia in response to ephrin-A5 stimulation (Fig 4.3e), a response seen in only $14.1 \pm 3.0\%$ of RGCs stimulated with ephrin-A5 alone ($p < 0.0001$; Student's T-Test for 2-tailed data of unequal variance). These data demonstrate that inhibiting ROCK activity prevents total collapse of RGC growth cones in response to stimulation with soluble ephrin-A5. They also provide strong evidence that Y27632 inhibits ephrin-A5-induced growth cone collapse by preventing loss of filopodia and axon retraction, but does not prevent ephrin-A5-induced loss of RGC lamellæ.

In response to soluble ephrin-A5, RGCs lose lamellæ entirely before losing filopodia and retracting (Chapter 3). It is therefore possible that Y27632 delays the cellular response to ephrin-A5 stimulation, revealing an intermediate stage of ephrin-A5-induced collapse in Y27632-treated RGC axons. To address this issue I have investigated the effect of Y27632 on the response of RGCs to 30 min stimulation with ephrin-A5. Longer incubations with anti-Fc antibodies (as a control) have no effect on the actin morphology of untreated (Fig 4.4a) and Y27632-treated (Fig 4.4b) RGCs. The actin morphology of RGCs treated with ephrin-A5-Fc for 30 min is similar to 10 min stimulation, since the RGCs lose both lamella and filopodia (Fig 4.4c). Following 30 min stimulation with ephrin-A5, Y27632-treated RGCs completely lose lamella, but still retain filopodia, demonstrating that ephrin-A5-Fc-induced loss of growth cone filopodia, is prevented by inhibiting ROCK, not simply delayed. Quantification of these experiments confirms this conclusion (Fig 4.4e). Y27632 significantly reduces the percentage of RGCs that totally collapse in response to ephrin-A5 ($21.7 \pm 1.0\%$) compared with RGCs stimulated with ephrin-A5 alone (73.5 ± 4.3 , $p < 0.004$; Student's T-Test for 2-tailed data of unequal variance). The majority ($59.7 \pm 4.3\%$) of Y27632-treated RGCs lose lamella but retain filopodia in response to ephrin-A5, compared to $9.7 \pm 4.3\%$ of RGC axons treated with ephrin-A5 alone ($p < 0.002$; Student's T-Test for 2-tailed data of unequal variance).

Although the persistence of RGC growth cone filopodia following ephrin-A5 addition correlates with lack of axon retraction (see Chapter 3), the above data do not conclusively prove that inhibiting ROCK activity prevents RGC axon retraction in

Figure 4.4 The ROCK inhibitor Y27632 does not prevent the loss of RGC growth cone lamellae in response to soluble 30 min stimulation with ephrin-A5, but does prevent loss of growth cone filopodia.



Y27632 does not delay ephrin-A5-induced loss of RGC filopodia. Phalloidin staining shows that control (a) and Y27632-treated (b) RGCs treated with anti-Fc control for 30 minutes have full growth cones with spread lamella and filopodia 30 minutes after addition of anti-Fc as control. 30 minutes stimulation with ephrin-A5 (1 μ g/ml) triggers loss of growth cone lamella and filopodia (c). Y27632 prevents the loss of filopodia, but not lamella, following 30 minutes stimulation with-ephrin-A5 (d). Quantification of the effect of Y27632 on ephrin-A5-treated RGCs demonstrates that pre-treatment with Y27632 (10 μ M) significantly reduces the percentage of RGCs that lose both filopodia and lamella (Total collapse) in response to 30 minutes stimulation with ephrin-A5-Fc (1 μ g/ml) (e, * $p < 0.004$, student's T-test for 2-tailed data of unequal variance). After 30 minutes stimulation with ephrin-A5 the majority (59.7 \pm 4.2%) of Y27632-treated RGCs lose lamella but not filopodia.

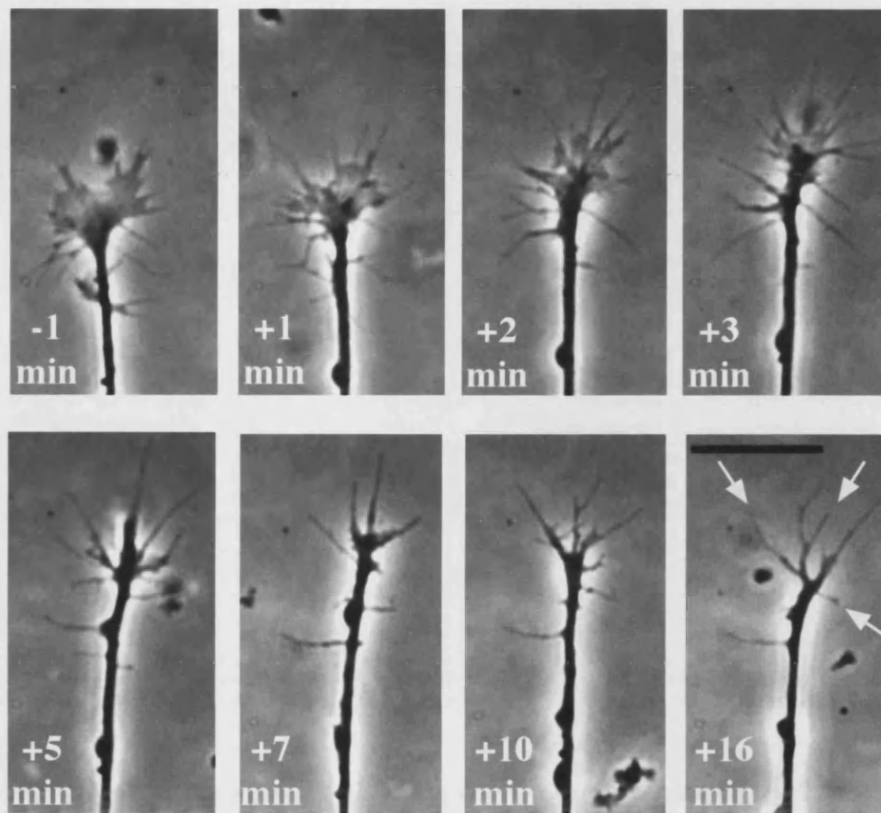
response to soluble ephrin-A5. Time-lapse stills showing the response of a Y27632-treated RGC axon to stimulation with $1\mu\text{gml}^{-1}$ ephrin-A5-Fc are shown in Fig 4.5. Following ephrin-A5 addition the RGC growth cone lamella is lost, although this is delayed compared to control RGC growth cones. On average Y27632-treated RGCs completely lose lamella 6.1 ± 1.4 min after ephrin-A5 addition ($n=6$) compared to 2.5 ± 0.5 min in the control situation ($n=6$, $p<0.001$; Student's T-Test for 2-tailed data of unequal variance). Despite the loss of lamellæ the RGC axon retains filopodia (Fig 4.5 arrows), and the axon fails to retract even after 16 min after ephrin-A5 addition (Fig 4.5). Of the 6 Y27632-treated RGC axons filmed, none retract within 15 min of ephrin-A5 stimulation, whereas all 6 control RGC axons retract within 3.5 min of ephrin-A5 addition ($p<0.003$, Fisher's exact test of independence). Taken together, the above data demonstrate that ROCK activity mediates ephrin-A-induced RGC axon retraction, but not loss of lamellæ, in response to both soluble and membrane-tethered ephrin-A.

4.2.3. The Src family kinase inhibitor PP2 does not prevent the RGC repulsive response to contact with an ephrin-A-expressing cell.

In order to investigate the role of Src family kinases in the RGC repulsive response to stimulation with ephrin-A, I have used PP2, a selective inhibitor of Src kinases (Hanke et al., 1996). Treatment of primary neurons in culture with $10\mu\text{M}$ PP2 completely inhibits phosphorylation of endogenous Src family substrates, and this concentration of PP2 has been used to implicate Src kinases in a variety of signalling pathways that influence the neuronal cytoskeleton (Crossthwaite et al., 2004; Hoffman-Kim et al., 2002; Manzerra et al., 2001; Suter and Forscher, 2001; Williamson et al., 2002). Co-cultures of retinal explants and Swiss 3T3 fibroblasts were therefore incubated with $10\mu\text{M}$ PP2 for 20-30 min prior to cell-cell contact.

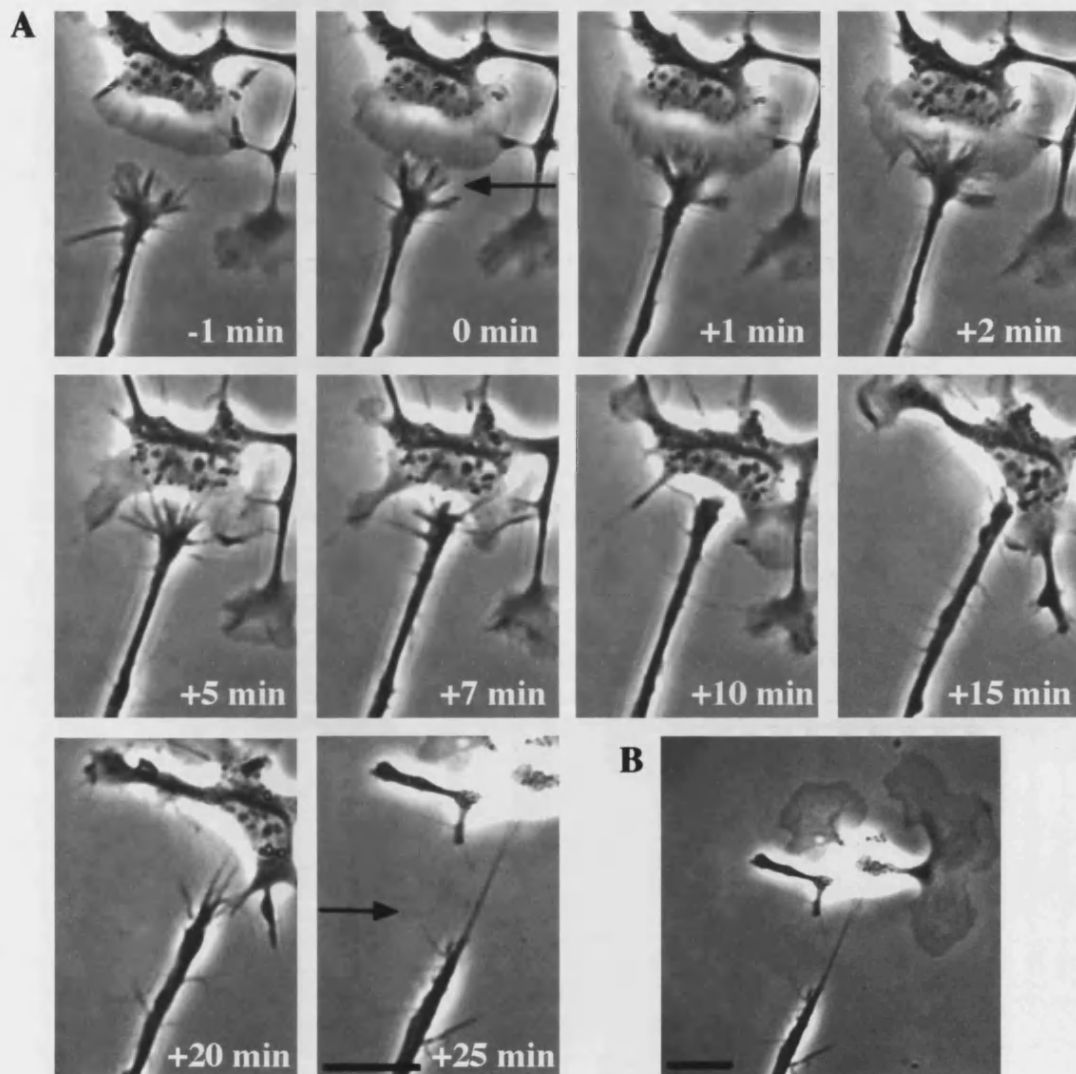
An example of the interaction between a RGC growth cone and an ephrin-A-expressing fibroblast following incubation with PP2 is shown in Fig 4.6. Pre-treatment with PP2 does not prevent the rapid loss of lamellæ in response to contact with the fibroblast; the lamella is completely lost by 7 min after contact (Fig 4.6A). I have included the image shown in Fig 4.6B, which is a larger field of view at 25 min after contact, to demonstrate that the repulsive RGC response is not a result of the fibroblast rounding up and detaching. Analysis of 15 RGC-fibroblast interactions

Figure 4.5 The ROCK inhibitor Y27632 prevents ephrin-A5-induced loss of RGC filopodia and axon retraction, but not loss of lamella.



Time-lapse stills showing that incubation with Y27632 (10 μ M) for 20 minutes prior to stimulation with ephrin-A5 (1 μ g/ml) prevents loss of RGC filopodia and axon retraction, but not loss of lamella. Times shown are relative to ephrin-A5 addition. The RGC growth cone lamella is completely lost by 5 minutes stimulation with ephrin-A5, but the axon does not retract within 16 minutes of stimulation with ephrin. The RGC axon retains filopodia following loss of lamella (see arrows). Scale bar = 20 μ m

Figure 4.6. PP2 does not prevent the RGC repulsive response to contact with an ephrin-A-expressing fibroblast.



A. Time-lapse stills showing that PP2 does not prevent loss of RGC lamella or axon retraction in response to contact with an ephrin-A-expressing fibroblast. PP2 (10 μ M) was added 20-30 minutes prior to cell contact and times shown are relative to initial contact. The RGC growth cone lamella is completely lost by 7 minutes after contact with the fibroblast. The RGC axon begins to retract after 20 minutes of contact (arrows), significantly later than in the control situation (see text). **B.** The Swiss 3T3 fibroblast is actively migrating following 25 minutes contact with the RGC axon. Scale bars = 20 μ m.

reveals that 80.0% of PP2-treated RGCs lose lamellæ within 10 min, compared with 76.7% of control RGCs ($p > 0.9$ Fisher's exact test for independence, see Table 4.2). The RGC axon in Fig 4.6 does not begin to retract after 20 min of contact (Fig 4.6 arrows). Pre-treatment with PP2 significantly delays the onset of RGC axon retraction induced by contact with an ephrin-A-expressing fibroblast (18.5 ± 6.1 min) compared with control (9.27 ± 6.0 min, $p < 0.002$; Student's T-Test for 2-tailed data of unequal variance). Despite this delay, inhibition of Src kinases with PP2 does not prevent RGC axon retraction induced by contact with an ephrin-A-expressing fibroblast, since 46.6% of PP2-treated axons retract within 20 min of contact compared with 43.3% of control axons ($p > 0.9$ Fisher's exact test for independence, see Table 4.2).

4.2.4. PP2 does not inhibit ephrin-A5-induced RGC growth cone collapse.

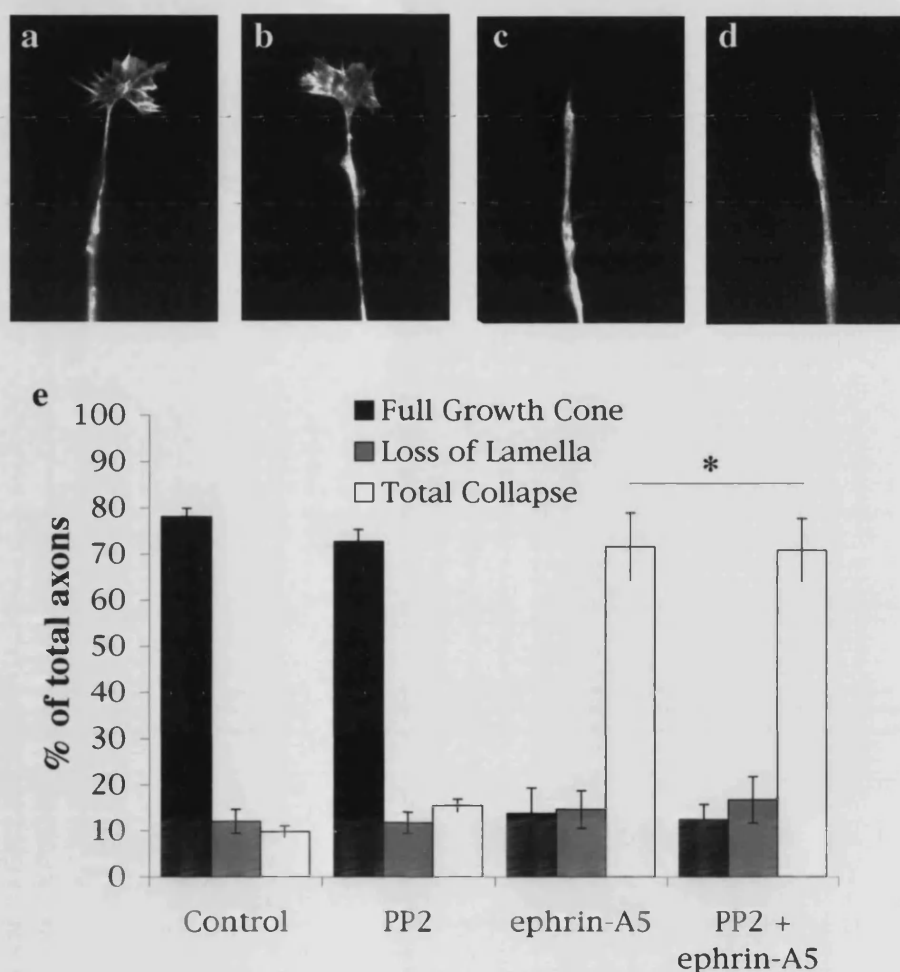
Addition of PP2 to the bathing medium of RGCs and Swiss 3T3 fibroblasts in co-culture could effect either cell type. In order to confirm that the action of PP2 specifically within RGCs has no effect on ephrin-A-induced growth cone collapse, RGCs were stimulated with soluble ephrin-A5 in the presence and absence of PP2.

Incubation with $10\mu\text{M}$ PP2 (Fig 4.7b) has no effect on the actin morphology of RGC growth cones compared with control (Fig 4.7a). PP2-treated RGC growth cones lose both lamellæ and filopodia in response to 10 min stimulation with $1\mu\text{gml}^{-1}$ ephrin-A5 (Fig 4.7d), similar to RGC growth cones treated with ephrin-A5 alone (Fig 4.7c). Quantification of these experiments (Fig 4.7e) confirms that pre-treatment with PP2 does not significantly reduce the percentage of RGC axons that totally collapse in response to ephrin-A5 ($70.8 \pm 6.9\%$) compared with ephrin-A5 alone ($71.6 \pm 7.3\%$, $p > 0.8$; Student's T-Test for 2-tailed data of unequal variance). These data suggest that Src family kinase activity is not required for the RGC repulsive response to stimulation with ephrin-A.

4.2.5. PP3 inhibits ephrin-A5-induced RGC growth cone collapse.

While investigating the effect of PP2 on ephrin-A5-induced RGC growth cone collapse I used PP3, a structural analogue of PP2 that does not inhibit Src kinases (Traxler et al., 1996), as a control. Intriguingly, pre-treatment with PP3 significantly

Figure 4.7. PP2 does not inhibit ephrin-A5-induced RGC growth cone collapse.



PP2 does not prevent ephrin-A5-Fc-induced loss of RGC growth cone lamella or filopodia. Phalloidin staining shows that control (a) and PP2-treated (10 μ M for 20 minutes {b}) RGCs have full growth cones with spread lamella and filopodia after addition of anti-Fc control for 10 minutes. Stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes triggers loss of growth cone lamella and filopodia (c) and this is unchanged by pre-treatment with PP2 (d). Quantification of the effect of PP2 on ephrin-A5-treated RGCs demonstrates that pre-treatment with PP2 (10 μ M) has no effect on the percentage of RGCs that lose both filopodia and lamella (Total collapse) in response to stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes (e, * $p > 0.8$, student's T-test for 2-tailed data of unequal variance).

Table 4.2.

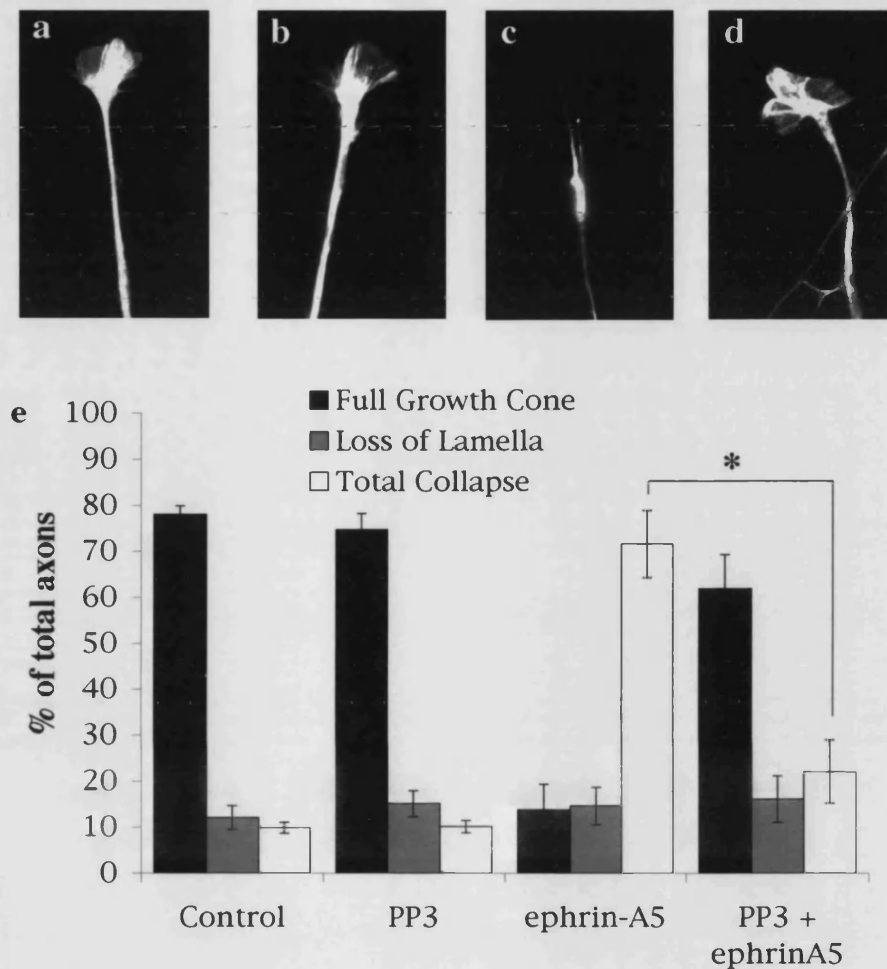
Cell type and treatment (n)	RGCs showing loss of lamella within 10 min of contact.	RGCs showing axon retraction within 20 min of contact.
Swiss 3T3 fibroblast (30)	76.7%	43.3%
Swiss 3T3 fibroblast + PP2 (15)	80.0% (12)	46.6% (7)
Swiss 3T3 fibroblast + PP3 (12)	33.3% (4)	8.3% (1)

inhibits RGC growth cone collapse induced by ephrin-A5-Fc (Fig 4.8). Incubation with 10 μ M PP3 alone (Fig 4.8b) has no effect on the actin morphology of RGC growth cones compared to control (Fig 4.8a) but prevents both loss of lamellæ and filopodia induced by ephrin-A5-Fc (Fig 4.8c and d). The inhibition of collapse is statistically significant; only $22.1 \pm 3.1\%$ of PP3-treated RGCs exhibit total collapse in response to stimulation with ephrin-A5, compared with $71.6 \pm 7.3\%$ of RGCs treated with ephrin-A5 alone ($p < 0.007$; Student's T-Test for 2-tailed data of unequal variance).

4.2.6. PP3 inhibits the RGC repulsive response to contact with an ephrin-A-expressing fibroblast.

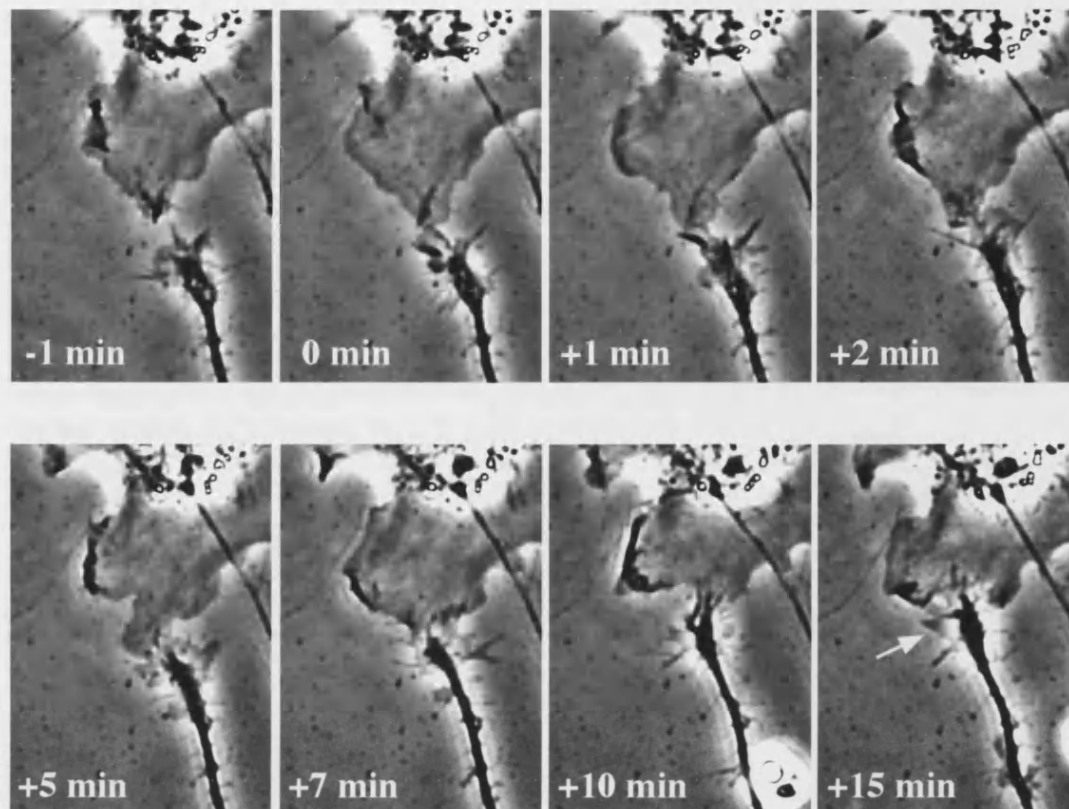
In order to determine whether the effects of PP3 are conserved in the response of RGCs to contact with ephrin-A-expressing fibroblast, RGCs and Swiss 3T3 fibroblasts in co-culture were incubated with 10 μ M PP3 for 20-30 min prior to cell-cell contact. Fig 4.9 shows an example of a RGC-fibroblast interaction following incubation with 10 μ M PP3. The RGC growth cone does not lose lamella upon contact with the ephrin-expressing fibroblast, but continues to advance. The lamella remains intact for greater than 15 min after initial contact with the fibroblast, and the RGC axon does not retract within this time. Only 33.3% of PP3-treated RGCs lose lamellæ within 10 min of contact with an ephrin-expressing fibroblast, compared to 76.7% of control RGCs ($p < 0.02$ Fisher's exact test for independence, see Table 4.2),

Figure 4.8. PP3 inhibits ephrin-A5-induced RGC growth cone collapse.



PP3 prevents ephrin-A5-Fc-induced loss of RGC growth cone lamella and filopodia. Phalloidin staining shows that control (a) and PP3-treated (10 μ M {b}) RGCs have full growth cones with spread lamella and filopodia after addition of anti-Fc control for 10 minutes. Stimulation with clustered ephrin-A5-Fc (1 μ g/ml) for 10 minutes triggers loss of growth cone lamella and filopodia (c). RGCs incubated with PP3 for 20 minutes prior to stimulation with ephrin-A5 retain both spread lamella and filopodia (d). Quantification of the effect of PP3 on ephrin-A5-treated RGCs demonstrates that pre-treatment with PP3 (10 μ M) significantly reduces the percentage of RGCs that lose both filopodia and lamella (Total collapse) in response to stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes (e, * $p < 0.007$, student's T-test for 2-tailed data of unequal variance).

Figure 4.9. PP3 prevents the RGC repulsive response to contact with an ephrin-A-expressing fibroblast.



Time-lapse stills showing that PP3 prevents loss of RGC lamella and axon retraction in response to contact with an ephrin-A-expressing fibroblast. PP3 (10 μ M) was added 20-30 minutes prior to cell contact and times shown are relative to initial contact. The RGC growth cone lamella is retained for more than 15 minutes after contact with the ephrin-expressing fibroblast (arrow), and the axon does not retract. Scale bar = 20 μ m.

and only 8.3% of PP3-treated RGC axons retract within 20 min, compared with 43.3% of control axons ($p < 0.04$ Fisher's exact test for independence, see Table 4.2).

4.2.7. Neither PP2 nor PP3 inhibit Eph receptor activity.

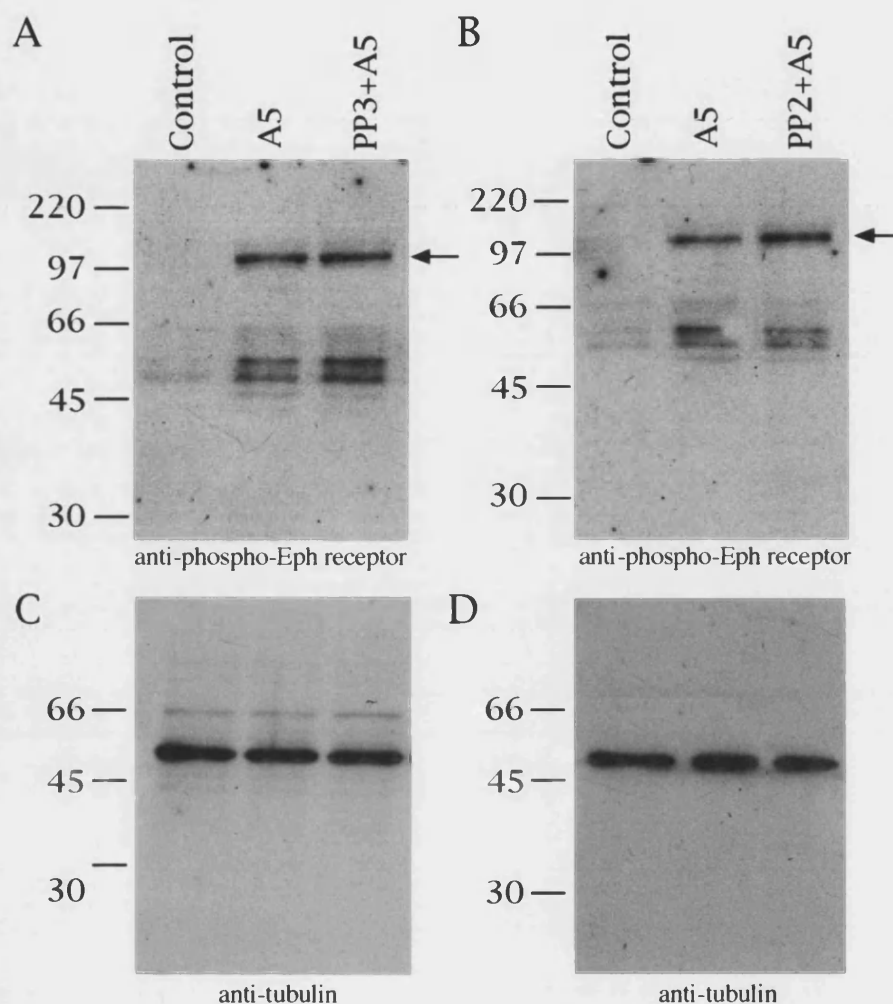
Of the known receptor tyrosine kinase families, the ErbB/EGF receptor family is most closely related to the Eph receptor family, and the EGFR inhibitor PD153035 has been shown to inhibit Eph receptor autophosphorylation (Ben-Shlomo et al., 2003; Sturz et al., 2004). Since PP3 is a known inhibitor of EGF receptor (Traxler et al., 1996) it is possible that PP3 inhibits ephrin-A-induced RGC repulsive responses by preventing Eph receptor activation. In addition PP2 has recently been shown to inhibit EphB4 activity *in vitro* and in primary endothelial cells (Sturz et al., 2004). I have therefore investigated whether PP2 or PP3 inhibit Eph receptor phosphorylation in RGCs in response to stimulation with ephrin-A5.

Isolated RGCs were incubated with 10 μ M PP2 or PP3 for 20 min prior to stimulation with 1 μ gml⁻¹ ephrin-A5 for 10 min. Ephrin-A5 treatment results in phosphorylation of Eph receptors, and this phosphorylation is not inhibited by incubation of RGCs with 10 μ M PP3 (Fig 4.10A) or PP2 (Fig 4.10B). This antibody specifically recognises Eph receptor phosphorylation at the conserved juxtamembrane tyrosines, which correlates with Eph kinase activity (Binns et al., 2000; Marston et al., 2003; Shamah et al., 2001). These data therefore demonstrate that PP3 and PP2 have no effect on ephrin-A5-stimulated Eph kinase activity in RGCs.

4.2.8. PP3 inhibits Semaphorin3A-induced DRG growth cone collapse.

Since PP3 does not inhibit ephrin-A5-stimulated Eph receptor activity, it must be inhibiting the ephrin-A-induced RGC repulsive response by some other mechanism. To determine whether the effects of PP3 are specific for the repulsive response induced by ephrin-A stimulation, I have investigated the effect of 10 μ M PP3 on growth cone collapse induced by a repulsive axon guidance cue unrelated to the ephrins, Semaphorin3A (Sema3A). E8 chick RGCs do not express the appropriate receptors for Sema3A, or collapse in response to Sema3A *in vitro* (Fournier et al., 2000; Takagi et al., 1995; Takahashi et al., 1998), whereas Sema3A induces growth cone collapse of chick dorsal root ganglion (DRG) neurons in culture Luo et al.,

Figure 4.10. Neither PP3 nor PP2 inhibits ephrin-A5-induced Eph receptor activation.



Isolated RGCs were pre-treated with either inhibitor (10 μ M) or carrier for 20 minutes. RGCs were then stimulated with 1 μ g/ml ephrin-A5-Fc (A5) or anti-Fc (Control) for 10 minutes. Cells were lysed with RIPA buffer and lysates were separated by SDS-PAGE and blotted with anti-phospho-Eph receptor antibody (A and B) to monitor receptor activity or anti-tubulin (C and D) to control for protein loading. Stimulation with ephrin-A5 for 10 minutes results in increased receptor phosphorylation (A and B, arrows), which is not inhibited by pre-treatment with PP3 (A) or PP2 (B).

1995). Fig 4.11 demonstrates that pre-treatment with PP3 significantly inhibits the percentage of DRG axons that lose both lamella and filopodia in response to stimulation with $5\mu\text{gml}^{-1}$ Sema3A (total collapse; $25.2 \pm 5.1\%$,) compared to Sema3A treatment alone ($62.0 \pm 4.3\%$, $p < 0.001$; Student's T-Test for 2-tailed data of unequal variance). These data suggest that PP3 has a general effect on neuronal signalling pathways that trigger growth cone collapse.

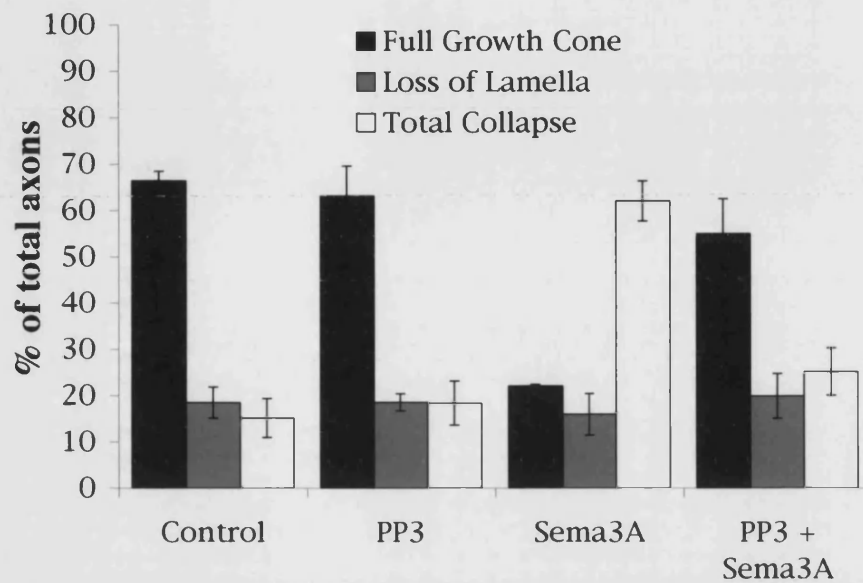
4.3. Discussion.

In Chapter 4 I have presented evidence that inhibition of ROCK activity in RGCs specifically prevents RGC axon retraction in response to stimulation with ephrin-A. Treatment with the ROCK-specific inhibitor Y27632 prevents RGC axon retraction, both in response to contact with an ephrin-A-expressing fibroblast, and to stimulation with soluble ephrin-A5-Fc. Loss of RGC growth cone lamellæ in response to membrane-tethered or soluble ephrin-A is not prevented by treatment with Y27632 however. The observation that ROCK inhibition does not prevent ephrin-induced loss of lamellæ is novel, since previous investigations into the effect of ROCK inhibition on ephrin-A-induced RGC growth cone collapse have not classified loss of RGC growth cone lamellæ separately from loss of filopodia (Cheng et al., 2003; Wahl et al., 2000). In addition I have presented evidence that the Src family kinase inhibitor PP2 does not prevent RGC growth cone collapse or axon retraction in response to stimulation with ephrin-A. Unexpectedly the structurally related compound PP3, which does not inhibit Src family kinases, prevents RGC growth cone collapse and axon retraction in response to both ephrin-A and Sema3A.

4.3.1. The ROCK inhibitor Y27632 prevents RGC axon retraction, but not loss of lamellæ, in response to stimulation with ephrin-A.

The data presented in this chapter demonstrate that treatment of RGCs with $10\mu\text{M}$ Y27632 prevents axon retraction in response to stimulation with soluble ephrin-A5-Fc (Figs 4.3, 4.4 and 4.5). Rho and ROCK have previously been implicated in the response of chick RGCs to soluble ephrin-A5 (Cheng et al., 2003; Wahl et al., 2000). Wahl and colleagues have reported that incubation of retinal explants with $10\mu\text{M}$ Y27632 significantly reduces the percentage of RGCs that exhibit growth cone collapse in response to stimulation with $1\mu\text{gml}^{-1}$ soluble ephrin-A5 (Wahl et al.,

Figure 4.11. PP3 inhibits Semaphorin3A-induced DRG growth cone collapse.



Pre-treatment with PP3 (10 μ M) for 20 minutes significantly reduces the percentage of DRGs that lose both filopodia and lamella (Total collapse) in response to stimulation with Sema3A (5 μ g/ml) for 10 minutes (e, * $p < 0.0008$, student's T-test for 2-tailed data of unequal variance).

2000). However the definition of growth cone collapse in the published study is complete loss of both filopodia and lamella; the authors did not investigate the loss of lamellæ versus filopodia, or ephrin-A5-induced axon retraction by time-lapse microscopy. The results presented in this chapter agree with the conclusion of the Wahl and colleagues, since incubation with Y27632 significantly reduces the percentage of RGC growth cones that lose both lamellæ and filopodia (“Total collapse”) in response to stimulation with soluble ephrin-A5 for 10 or 30 min (Figs 4.3 and 4.4). I have also extended the conclusions of the published study by investigating the response to ephrin-A5 dynamically and in greater detail, to demonstrate that the loss of RGC growth cone lamellæ in response to ephrin-A5 is not inhibited by Y27632, while loss of growth cone filopodia and axon retraction are (Figs 4.3, 4.4, 4.5).

The specific effect of ROCK inhibition on loss of filopodia and axon retraction is conserved during the response of RGC growth cones to contact with an ephrin-A-expressing fibroblast, arguably a more relevant assay for the study of Eph receptor signalling during axon guidance (see Chapter 3). During the course of this thesis a related study was published investigating the role of ROCK activity on the behaviour of RGC growth cones following contact with posterior tectal neurons (Thies and Davenport, 2003). The authors did not address whether the RGC growth cone collapse and axon retraction induced by contact with a tectal neuron is due to Eph receptor-ephrin interaction, but report that Y27632 reduces the percentage of RGCs that lose both lamellæ and filopodia following cell-cell contact, as well as the incidence of axon retraction, confirming the data I have described in Chapter 4 (Thies and Davenport, 2003).

ROCK can increase MLC phosphorylation, and thus stimulate its ATPase activity, directly as well as indirectly by phosphorylating and inactivating MLCP (Amano et al., 1996; Kimura et al., 1996). These effects are potentiated by the presence of active Rho (Amano et al., 1996; Kimura et al., 1996), and thus ROCK activity is thought to mediate the formation of RhoA-induced stress fibres, regulating the contractility of non-muscle cells (Fukata et al., 2001). In neuronal cells the Rho-ROCK-MLC pathway has been shown to mediate axon retraction. Microinjection of constitutively active RhoA or ROCK into neuronal cell lines is sufficient to cause rapid withdrawal of neurites, as is injection of a MLC mutant, which mimics phosphorylation by

ROCK (Amano et al., 1998; Katoh et al., 1998; Kozma et al., 1997). ROCK activity is sufficient to trigger neurite retraction under conditions when Rho activity is inhibited, demonstrating that ROCK acts downstream of Rho during axon retraction (Katoh et al., 1998). It must be noted that, although Wahl and colleagues have demonstrated that treatment of E7 chick RGCs with 10 μ M Y27632 for 1 hour is sufficient to completely inhibit ROCK activity in these neurons, I have not directly addressed whether ROCK activity is inhibited under the conditions used in this chapter. Y27632 also inhibits the Rho-dependent kinases PRK1 and PRK2 at similar concentration to ROCK (Amano et al., 1999; Davies et al., 2000). To date there is no evidence that these kinases play a role in growth cone collapse, although they are known regulators of the cytoskeleton.

The Rho family GEF Ephexin is expressed in RGC growth cones, and when co-expressed with EphA4 in COS cells, Ephexin increases the proportion of cells that exhibit stress fibres, indicative of Rho activation (Shamah et al., 2001). Although the authors did not investigate the effect of Eph receptor activation on Ephexin's GEF activity towards Rho, the observation that dominant negative Ephexin inhibits ephrin-A-induced growth cone collapse lead them to the conclusion that ephrin-A-induced increase in Rho activity in RGCs is mediated by Ephexin (Shamah et al., 2001). Inhibition of Rho and ROCK result in similar levels of inhibition of ephrin-A5-induced growth cone collapse (Wahl et al., 2000) suggesting that ROCK is the major Rho effector during neuronal repulsive responses to ephrin-A. Since ephrin-A5 stimulation of RGCs leads to an increase of active Rho (Wahl et al., 2000) and inhibition of ROCK activity prevents ephrin-A-induced RGC axon retraction (Figs 4.1, 4.2, 4.5, Table 4.1) it is likely that ephrin-A stimulation activates the Rho-ROCK-MLC pathway to trigger axon retraction. The Rho-ROCK-MLC pathway has been implicated in axon retraction induced by G-protein coupled receptor agonists such as lysophosphotidic acid (LPA). LPA treatment of NE1-115 neuroblastoma cells increases both Rho activity and ROCK-dependent phosphorylation of MLC (Hirose et al., 1998; Kranenburg et al., 1999), and inhibition of Rho or ROCK prevents LPA-induced neurite retraction in a number of neuronal cell lines (Amano et al., 1998; Hirose et al., 1998; Jalink et al., 1994; Tigyi et al., 1996). In addition there is evidence that repulsive axon guidance cues other than ephrin-A stimulate the Rho-ROCK pathway to trigger growth cone collapse. For example Stimulation of Robo-expressing cells with Slit, the ligand for Robo, induces activation of Rho (Wong et al., 2001). Expression of dominant negative Rho in *Drosophila* significantly enhances the aberrant CNS axon guidance phenotype in both Slit and Robo mutants (Fan et al., 2003; Fritz and VanBerkum, 2002), implying that increased Rho activation may be required for the repulsive response to Slit/Robo interaction, although to date this has not been tested directly. Activation of PlexinB molecules, receptors for the class 4 Semaphorins, increases the

cellular levels of active Rho (Aurandt et al., 2002; Hu et al., 2001; Oinuma et al., 2003; Perrot et al., 2002), and expression of a dominant negative Rho GEF inhibits Semaphorin4D-induced growth cone collapse of hippocampal neurons (Swiercz et al., 2002). By contrast growth cone collapse induced by Sema3A involves Rac signalling (Jin and Strittmatter, 1997; Kuhn et al., 1999; Vastrik et al., 1999) but is independent of the Rho-ROCK pathway (Arimura et al., 2000; Goshima et al., 1995). Together these data suggest that activation of the Rho-ROCK-MLC pathway is a mechanism by which many, but not all, inhibitory axon guidance cues effect their repulsive neuronal responses.

4.3.2. The ROCK inhibitor Y27632 prevents loss of RGC growth cone filopodia.

Treatment of RGCs with Y27632 prevents the loss of filopodia in response to soluble and membrane tethered ephrin-A stimulation. As discussed in Chapter 3 the RGC repulsive response to ephrin-A is sequential, and the RGC growth cone filopodia appear to be dragged away as the axon retracts. If axon retraction is directly responsible for the loss of RGC growth cone filopodia, it is possible that inhibition of axon retraction by Y27632 is sufficient to prevent ephrin-A-induced loss of filopodia.

The observations that expression of constitutively active ROCK leads to down-regulation of integrin adhesions in leukocytes, and that Y27632-treatment stimulates the formation of new vinculin-positive focal complexes in fibroblasts (Rottner et al., 1999b; Worthylake et al., 2001) could provide an additional explanation for ROCK inhibition preventing ephrin-A-induced loss of filopodia. Growth cones of neurons on laminin exhibit cell-matrix adhesions that contain many of the same proteins as focal complexes seen in fibroblasts (Renaudin et al., 1999). Vinculin-positive adhesions are seen on growth cone filopodia, but not lamella (Renaudin et al., 1999). ROCK activity may downregulate these filopodial adhesions during ephrin-A-induced growth cone collapse and axon retraction, and therefore Y27632 would increase these adhesions, preventing the loss of filopodia, but not lamella.

4.3.3. The ROCK inhibitor Y27632 delays ephrin-A-induced loss of RGC lamellae.

Although inhibition of ROCK activity does not prevent loss of RGC lamella in response to contact with an ephrin-A-expressing fibroblast, or soluble ephrin-A5, there is a significant delay in the loss of lamellæ of Y27632-treated RGC growth cones following stimulation with ephrin-A. One potential explanation for this observation is that ROCK activity contributes to loss of lamellæ through the regulation of actomyosin contraction in the lamella itself. However, treatment of goldfish RGCs with a peptide inhibitor of MLCK leads to a dramatic reduction of lamellæ protrusion (Schmidt et al., 2002), suggesting that the Rho-ROCK-MLC pathway does not act to limit lamellæ protrusion, at least during axon outgrowth.

Recently it has been demonstrated that Y27632 increases Rac activation in fibroblasts treated with LPA (Tsuji et al., 2002). Soluble ephrin-A stimulation of RGCs leads to a transient decrease in Rac activity (Jurney et al., 2002; Wahl et al., 2000), and it is therefore possible that inhibition of ROCK antagonises this inhibition of Rac, allowing Y27632-treated RGC growth cone lamellæ to persist for longer during ephrin-A stimulation. Since expression of constitutively active Rac in DRG neurons does not inhibit ephrin-A-induced growth cone collapse (Jurney et al., 2002), Y27632-mediated dis-inhibition of Rac activity would not be predicted to prevent loss of RGC lamellæ in response to stimulation with ephrin-A, which agrees with the data presented in this chapter.

ROCK can activate LIM kinase, and expression of constitutively active LIM kinase in DRG neurons results in growth cones that specifically lose lamellæ (Aizawa et al., 2001; Ohashi et al., 2000; Sumi et al., 2001). Treatment of RGC with Y27632 may therefore partially inhibit LIM kinase-mediated loss of lamellæ although LIM kinase has not been implicated in ephrin-A-induced growth cone collapse. However, LIM kinase activity is required for Sema3A-induced growth cone collapse, which is a ROCK-independent event (Aizawa et al., 2001; Arimura et al., 2000; Goshima et al., 1995). LIM kinase is also activated by the Rac/Cdc42 effector, PAK (Edwards et al., 1999), and together with the observation that ephrin-A stimulation leads to activation of Rho but inhibition of Rac, this makes predicting the effect, if any, of LIM kinase on ephrin-A-induced RGC lamellæ loss difficult. Overall the data presented in this

chapter strongly suggest that ROCK activity is critically involved in ephrin-induced axon retraction, but another pathway must be involved in ephrin-induced loss of lamellæ.

4.3.4. PP2 delays axon retraction in response to contact with an ephrin-expressing fibroblast.

Incubation with PP2 does not prevent loss of RGC growth cone lamellæ and axon retraction in response to ephrin-A stimulation. However it does significantly delay the onset of RGC axon retraction following contact with an ephrin-A-expressing fibroblast. It is possible that the PP2-dependent delay in axon retraction is a result of Src kinase regulation of cell-matrix adhesions.

Migrating fibroblasts lacking Src, Fyn and Yes assemble new cell-matrix contacts at the leading edge normally, but show much reduced adhesion disassembly, assayed by loss of fluorescent paxillin (Webb et al., 2004). Expression of kinase dead Src, or treatment with PP2 also reduces the rate of adhesion disassembly (Webb et al., 2004). Paxillin-containing adhesions on the filopodia of neuronal growth cones plated on laminin also contain FAK (Renaudin et al., 1999). As discussed in section 1.6.2, Src activity is thought to mediate adhesion disassembly by degradation of FAK (Carragher et al., 2001; Carragher et al., 2003; Fincham et al., 1995). Fyn is found co-localised with FAK in filopodia-substrate adhesions (Renaudin et al., 1999), and by analogy with the role of Src family kinases in non-neuronal cells, may act to disassemble these paxillin-containing adhesions. Since EphA activation in non-neuronal cells can result in an increase in Fyn activity (Sharfe et al., 2003) it is possible that incubation of RGCs with PP2 inhibits ephrin-A-induced Fyn activity and subsequent disassembly of matrix adhesions along the filopodia. Integrin-mediated adhesions containing FAK and paxillin are also present on the axons of chick retinal neurons grown on laminin (de Curtis and Malanchini, 1997) and it is therefore possible that PP2 might antagonise ephrin-A-induced loss of axonal adhesions. These effects on matrix adhesions may delay axon retraction until the Rho-ROCK pathway is stimulated sufficiently to overcome the filopodial adhesion. In support of this hypothesis, ephrin-A stimulation of non-neuronal cells does reduce cell-matrix adhesion (Miao et al., 2000), although the role of Src family kinases in ephrin-A-induced loss of adhesion has not been addressed.

It should be noted that a recently published study reports that PP2 inhibits ephrin-A5-induced RGC growth cone collapse (Wong et al., 2004). Wong and colleagues include temporal RGCs in their study, whereas I have only used nasal RGCs. Temporal RGCs express higher levels of EphA3 than nasal RGCs (Cheng et al., 1995; Connor et al., 1998; Monschau et al., 1997) and express a higher level of “available” EphA4 (Hornberger et al., 1999). It is therefore possible that different Eph receptors mediate the collapse response reported by Wong and colleagues, and described in this thesis. Temporal RGCs are more sensitive to ephrin-A5 than nasal RGCs (Nakamoto et al., 1996) and I find that the collapse response of nasal RGCs saturates at around $1\mu\text{gml}^{-1}$, which raises the possibility that $25\mu\text{gml}^{-1}$ ephrin-A5 (as used by Wong and colleagues) may activate additional, low-affinity Eph receptors. It is therefore possible that the signalling pathways activated by stimulation of a mixture of nasal and temporal RGCs with $25\mu\text{gml}^{-1}$ ephrin-A5 (Wong et al., 2004) differ from those activated by treatment of nasal RGCs with $1\mu\text{gml}^{-1}$ ephrin-A5 (this chapter). This would account for the former being sensitive to PP2, especially at a concentration of $25\mu\text{M}$ (Wong et al., 2004), while the latter are not sensitive to $10\mu\text{M}$ PP2 (Fig 4.7). The use of PP2 at $25\mu\text{M}$ also has potential implications for the conclusion drawn by Wong and colleagues, that Src family kinase activity is required for ephrin-A5-induced RGC growth cone collapse. $10\mu\text{M}$ PP2 inhibits ligand-induced EphB activity (Sturz et al., 2004). I have shown that $10\mu\text{M}$ PP2 does not inhibit Eph receptor activity in response to $1\mu\text{gml}^{-1}$ ephrin-A5 (Fig 4.10), but $25\mu\text{M}$ PP2 may interfere with the activity of Eph receptors activated by $25\mu\text{gml}^{-1}$ ephrin-A5. This was not addressed in the published study (Wong et al., 2004).

One example of a PP2-sensitive signalling intermediate that might be differentially activated by different levels of Eph receptor activity is p190RhoGAP. p190RhoGAP has preferential GAP activity for Rho *in vitro* (Ridley et al., 1993) and is expressed at high levels in the developing mammalian nervous system (Brouns et al., 2001). Src and Fyn can directly phosphorylate p190RhoGAP *in vitro* and *in vivo* (Brouns et al., 2001; Haskell et al., 2001; Roof et al., 1998; Wolf et al., 2001), and there is evidence that this phosphorylation event mediates p190RhoGAP-induced loss of stress fibres (Haskell et al., 2001). Together these observations suggest that in the nervous system Src and Fyn may down-regulate Rho signalling via increased p190RhoGAP activity, and this is one explanation for the observation that while inhibition of the Rho-

ROCK pathway with Y27632 prevents the ephrin-A-induced axon retraction reported in this chapter, PP2 does not have the same effect. However, Eph receptors also have the potential to increase p190RhoGAP phosphorylation via Src family kinases. Src and Fyn can activate LMW-PTP and cause an associated decrease in p190RhoGAP phosphorylation (Bucciantini et al., 1998; Rigacci et al., 1996; Tailor et al., 1997). Since there is a correlation between p190RhoGAP phosphorylation and reduced Rho activity (Arthur et al., 2000), it is possible that Src or Fyn activation could contribute to increased Rho activation via LMW-PTP-mediated reduction in p190RhoGAP activity. A variety of Eph receptors have been shown to activate Src and Fyn (Sharfe et al., 2003; Steinle et al., 2002; Takasu et al., 2002; Vindis et al., 2003), and it is therefore possible that stimulation of RGCs with $25\mu\text{gml}^{-1}$ ephrin-A5 activates a Src-dependent increase in Rho activity, mediated by Eph receptors that are not activated by $1\mu\text{gml}^{-1}$ ephrin-A5. This might explain why growth cone collapse in response to $25\mu\text{gml}^{-1}$ ephrin-A5 is sensitive to PP2 (Wong et al., 2004), while loss of lamella and axon retraction in response to contact with an ephrin-A-expressing fibroblast or $1\mu\text{gml}^{-1}$ ephrin-A5 are not.

I have not addressed whether Src family kinase activity is inhibited in RGCs following incubation with $10\mu\text{M}$ PP2. Incubation with this concentration of PP2 significantly delays RGC axon retraction following contact with an ephrin-expressing fibroblast, but does not prevent it, which presents the possibility that inhibition of Src family kinases by $10\mu\text{M}$ PP2 may be incomplete. Although treatment of *Aplysia* neurons with $10\mu\text{M}$ PP2 significantly reduces repulsive growth cone responses, further inhibition can be achieved with $25\mu\text{M}$ PP2 (Suter and Forscher, 2001). I have been unable to determine the effect of higher concentrations of PP2 on ephrin-A-induced growth cone collapse and axon retraction, because PP2 at concentrations higher than $10\mu\text{M}$ induce RGC growth cone collapse and axon retraction independent of ephrin-A stimulation. $10\mu\text{M}$ PP2 is sufficient to inhibit phosphorylation of Src family substrates in other types of primary neuron in culture (Crossthwaite et al., 2004; Manzerra et al., 2001), and therefore the results presented in this chapter suggest that Src family kinases do not play a major role in ephrin-A-induced RGC repulsive responses. However in order to confirm this conclusion I would have to investigate the effect of other pharmacological inhibitors of Src family kinases on ephrin-A-induced growth cone collapse and axon retraction. SU 6656 has

different non-specific effects to PP2, and is therefore a good candidate for confirming that Src family kinase activity is not required for ephrin-A-induced growth cone collapse and axon retraction (Bain et al., 2003; Blake et al., 2000)

4.3.5. PP3 prevents the repulsive RGC response to stimulation with ephrin-A.

Surprisingly the structural analogue of PP2, PP3, has a dramatic inhibitory effect on the growth cone collapse and axon retraction induced by stimulation with soluble and membrane-tethered ephrin-A (Fig 4.8, 4.9). PP3 must therefore influence a major mediator of the RGC repulsive response. One obvious possibility is that PP3 interferes with ephrin-A-induced Eph receptor activation. This was an intriguing possibility as to date there are no pharmacological tools available that inhibit EphA kinase activity, but incubation of isolated RGCs with 10 μ M PP3 for 20 min has no effect on the level of Eph receptor phosphorylation induced by stimulation with ephrin-A5-Fc (Fig 4.10). In agreement with this, the effect of PP3 is not specific to Eph receptor-mediated growth cone collapse, because 10 μ M PP3 also significantly inhibits DRG growth cone collapse induced by Sema3A (Fig 4.11).

PP3 inhibits casein kinase 1 delta (CK1 δ) *in vitro* (Bain et al., 2003) and this enzyme has recently been shown to increase phosphorylation of the microtubule associated protein tau *in vivo* (Li et al., 2004). Interestingly tau phosphorylation is greatly increased in RGCs following stimulation with ephrin-A5, and in DRGs after Sema3A stimulation (Cheng et al., 2003; Sasaki et al., 2003). Pharmacological inhibition of Cdk5, which phosphorylates tau, inhibits growth cone collapse induced by both guidance cues, although the authors did not investigate whether neuronal tau phosphorylation is inhibited under these conditions (Cheng et al., 2003; Sasaki et al., 2003). PP3 significantly inhibits both ephrin-A5 and Sema3A-induced growth cone collapse of RGCs and DRGs respectively. One possibility therefore is that PP3 inhibits CK1 δ in these neurons, preventing ephrin-A or Sema3A-induced tau phosphorylation, and interfering with the microtubule rearrangements that occur during growth cone collapse (Fan et al., 1993; Meima et al., 1997a; Meima et al., 1997b). However, PP2 also inhibits CK1 δ *in vitro*, with approximately 8-fold higher potency compared to PP3 (Bain et al., 2003) yet PP2 does not prevent RGC growth cone collapse or axon retraction in response to ephrin-A.

Other kinases known to be inhibited by PP3 are EGF receptor and Bmx, but again these kinases are also inhibited by PP2 with a higher potency (Traxler et al., 1996; J.A. Cooper, personal communication). It is therefore difficult to interpret the results presented in this chapter demonstrating that PP3 prevents both RGC growth cone collapse and axon retraction in response to stimulation with ephrin-A. The observation that PP3 also inhibits Sema3A-induced growth cone collapse, known to involve a different mechanism to ephrin-A-induced growth cone collapse, could imply that PP3 interferes with common cytoskeletal machinery, and therefore is non-specific with regards to ephrin-A-induced growth cone collapse.

Chapter 5. The role of Abl in ephrin-A-induced RGC repulsive responses.

5.1. Introduction.

In Chapter 4 I have presented evidence that ROCK mediates ephrin-A-induced RGC axon retraction, but not loss of RGC growth cone lamellæ, and therefore other signalling molecules must mediate this aspect of the ephrin-A-induced RGC repulsive response. As discussed in section 1.7 the non-receptor tyrosine kinase Abl, implicated in axon guidance decisions *in vivo*, induces rearrangement of the neuronal actin cytoskeleton, and regulates the activity of Mena, a member of the Ena/VASP family of proteins that regulate actin dynamics within the lamella (Bear et al., 2002; Hsouna et al., 2003; Wills et al., 1999; Wills et al., 2002; Woodring et al., 2002). Abl has been shown to interact with EphA4, which is expressed by chick RGC growth cones *in vitro*, and is required for the repulsive response of nasal RGC axons to ephrin-A5 (Monschau et al., 1997; Walkenhorst et al., 2000; Yu et al., 2001). Abl kinase can be activated by direct phosphorylation of its activation loop by exogenous kinases, and Eph receptors have been shown to directly phosphorylate Abl (Yu et al., 2001). It is therefore possible that EphA-activation stimulates Abl kinase activity, and thus induces the actin rearrangements that underlie ephrin-A-induced growth cone collapse.

In *Drosophila*, D-abl mutants show defects in CNS axon pathfinding, and this phenotype is suppressed by mutations in Ena, the *Drosophila* homologue of Mena (Hsouna et al., 2003; Wills et al., 1999; Wills et al., 2002). Mena is an Abl substrate, and is also required for correct axon guidance during development (Lanier et al., 1999; Tani et al., 2003). Mena-deficient mice show defects in the guidance of cortical axons that would normally form the corpus callosum; these axons reach the midline but do not cross to the contraeral cortex (Lanier et al., 1999). This phenotype is very similar to that seen in EphA5 mutant mice (Hu et al., 2003), although to date no genetic or biochemical interactions have been reported between Mena and Eph receptors.

In mammalian cells Mena is localised to the very edge of protruding lamella (Nakagawa et al., 2001; Rottner et al., 1999a). Ena/VASP family proteins at the leading edge of fibroblast lamellipodia directly influence the organisation of actin filaments within the lamella, and the accumulation of Ena/VASP proteins at the leading edge directly correlates with the rate of lamella protrusion (Bear et al., 2002; Rottner et al., 1999a). Mena is highly enriched in the growth cone lamella of primary neurons in culture, and since Mena-deficient neurons extend axons normally *in vitro* (Lanier et al., 1999), Mena is therefore a good candidate for regulating actin dynamics in the growth cone lamella in response to axon guidance cues.

Genetic evidence suggests that D-abl antagonises Ena function. If Abl negatively regulates Mena function in lamella protrusion, it is possible that Abl links EphA activity to loss of RGC lamella. In this chapter I have used a pharmacological inhibitor of Abl kinase activity, STI571 (Gleevec, Novartis), to investigate the role of Abl in ephrin-A-induced growth cone collapse. I have determined the effect of this inhibitor on the behaviour of RGC axons following stimulation with ephrin-A, and have demonstrated that STI571 prevents both the loss of RGC lamellæ and axon retraction in response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast, and following addition of ephrin-A5-Fc. The effect of STI571 is specific to Eph receptor signalling, as STI571 does not inhibit Sema3A-induced DRG growth cone collapse. In addition I have demonstrated an interaction between active Eph receptors and endogenous Abl and Mena in COS cells, and shown that STI571 disrupts this complex.

5.2. Results.

5.2.1. The Abl kinase inhibitor STI571 prevents the repulsive response to contact with an ephrin-A-expressing cell.

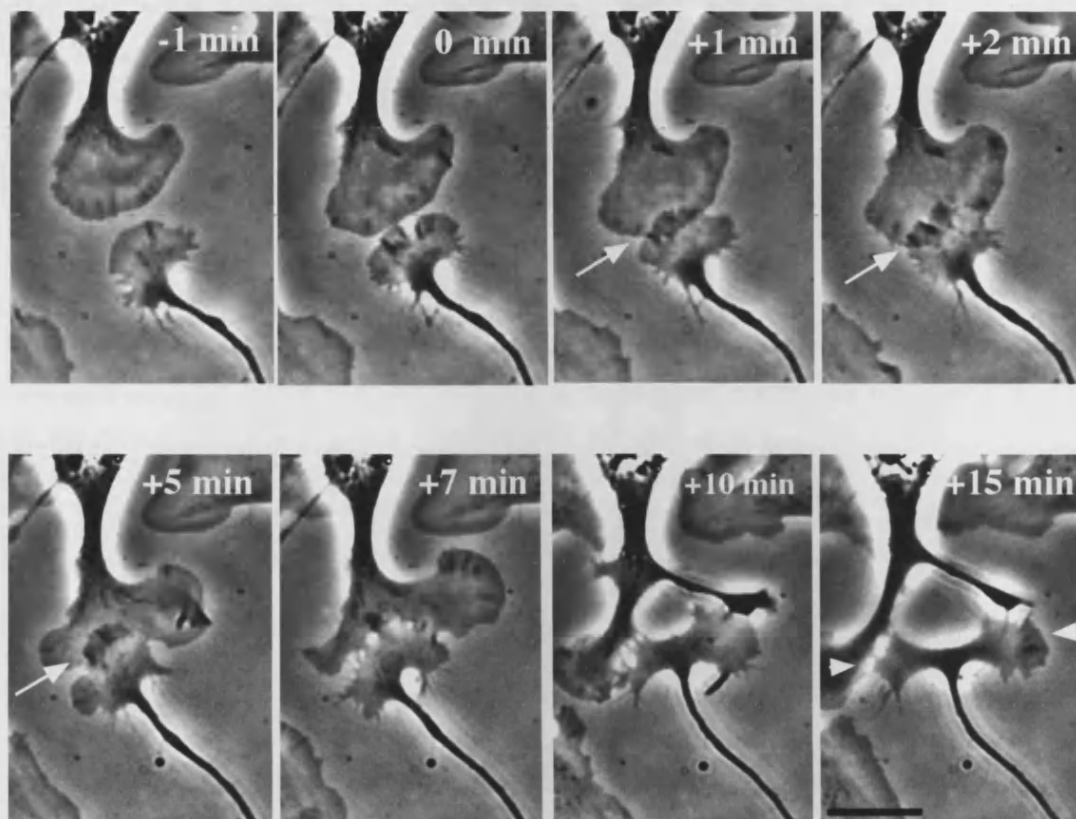
To determine the role of Abl kinase in the repulsive response of RGCs to ephrin-A stimulation I have investigated the effect of STI571 on the behaviour of RGCs following contact with Swiss-3T3 fibroblasts, which endogenously express ephrin-As (see Chapter 3). Co-cultures of retinal explants and Swiss-3T3 fibroblasts were incubated with 10 μ M STI571 for 20-30 min prior to cell-cell contact. An example of the interaction between a RGC growth cone and a Swiss-3T3 fibroblast after STI571

treatment is shown in Fig 5.1 (see also movie 5.1). Following contact with the fibroblast the RGC growth cone continues to advance, retaining a large spread lamella for more than 15 min after initial contact (Fig 5.1, movie 5.1). At later time-points the RGC growth cone shown in Fig 5.1 preferentially extends lamella at sites of cell-cell contact (Fig 5.1, arrowheads). As discussed in chapter 3, control RGC-Swiss-3T3 fibroblast interactions result in rapid loss of RGC growth cone lamellæ and axon retraction (Fig 3.2). Fig 5.1 demonstrates that incubation with the Abl kinase inhibitor STI571 prevents both these aspects of the RGC repulsive response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast.

Analysis of 15 STI571-treated RGC-fibroblast interactions reveals that STI571 significantly reduces the percentage of RGCs that lose lamellæ and retract following contact with a Swiss-3T3 fibroblast. Only 13.3% of STI571-treated RGCs lose lamella within 10 min of contact with a Swiss-3T3 fibroblast, compared with 76.7% of control RGCs (see Table 5.1, $p < 0.0001$; Fisher's exact test for independence) and none of the STI571-treated RGC-fibroblast interactions induce RGC axon retraction (see Table 5.1, $p < 0.002$; Fisher's exact test for independence). These data demonstrate that incubation with STI571 prevents the loss of RGC lamellæ and axon retraction induced by contact with an ephrin-A expressing Swiss-3T3 fibroblast.

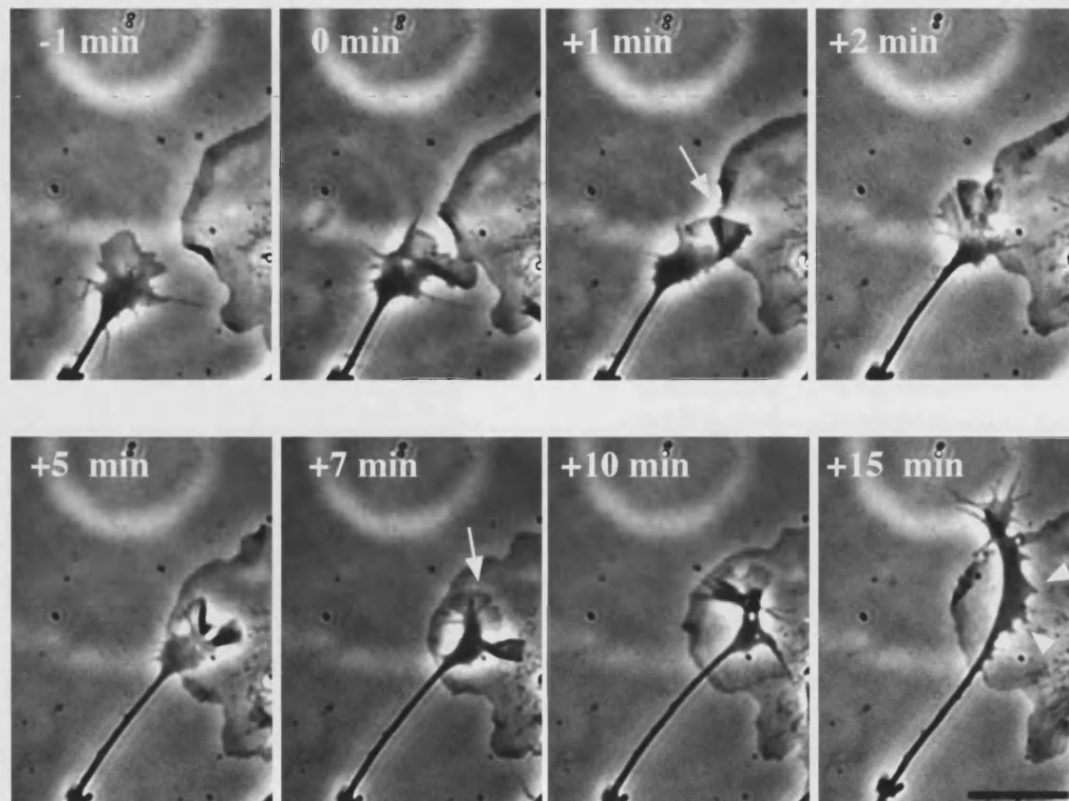
In the example shown in Fig 5.1 the lamella of the RGC growth cone advances a short distance across the lamella of the Swiss-3T3 fibroblast and the two lamellæ overlap for more than 5 min before the fibroblast withdraws slightly (Fig 5.1 arrows). The majority (69.2%) of STI571-treated RGCs that do not lose growth cone lamellæ in response to contact extend lamellæ over the surface of the fibroblast for more than 5 min after contact ($n=13$). Interestingly none of the control RGCs that do not lose lamellæ in response to contact with a Swiss-3T3 fibroblast extend growth cone lamellæ over the fibroblast surface ($n=5$), suggesting that the lamellæ overlap shown in Fig 5.1 is a result of STI571 treatment. An extreme example of an STI571-treated RGC extending growth cone lamella over the surface of a Swiss-3T3 fibroblast is shown in Fig 5.2 (see also movie 5.2). Following contact the RGC growth cone lamella extends over the lamella of the fibroblast (Fig 5.2, arrow) and the growth cone advances so that the RGC axon extends across the fibroblast surface (Fig 5.2, movie 5.2). At later time-points, as the RGC growth cone reaches the substrate on the far side of the fibroblast, the RGC protrudes lamella along the portion of the axon

Figure 5.1. STI571 prevents the RGC repulsive response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast.



Time-lapse stills showing that STI571 inhibits loss of RGC growth cone lamella and axon retraction in response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast. STI571 (10 μ M) was added 20-30 minutes prior to cell contact and times shown are relative to initial contact. The RGC growth cone lamella is not lost following contact with the fibroblast, and at early time-points the RGC growth cone lamella extends over the surface of the fibroblast (+1, +2 and +5 min, arrows). In addition STI571 prevents RGC axon retraction in response to contact with the fibroblast, and the axon continues to advance across the substrate. Note that as the fibroblast begins to withdraw, the RGC growth cone preferentially extends lamella at the remaining sites of contact with the fibroblast (+15 min, arrowheads). Scale bar = 20 μ m.

Figure 5.2. STI571 treatment may not result in a neutral response of RGCs to contact with an ephrin-A-expressing Swiss-3T3 fibroblast.



Time-lapse stills showing that STI571 inhibits loss of RGC lamella and axon retraction in response to contact with an ephrin-A-expressing Swiss-3T3 fibroblast. STI571 (10 μ M) was added 20-30 minutes prior to cell contact and times shown are relative to initial contact. The RGC growth cone lamella is not lost following contact with the fibroblast but extends lamella over the surface of the fibroblast (+1 min, arrow) and then continues to advance so that the entire growth cone is in contact with the fibroblast (+7 min, arrow). The axon advances across the Swiss-3T3 fibroblast and back onto the substrate, protruding lamella along the distal portion of the axon that is still in contact with the cell (+15 min, arrowheads). Scale bar = 20 μ m

Table 5.1

Cell type and treatment (n)	RGCs showing loss of lamella within 10 min of contact (n).	RGCs showing axon retraction within 20 min of contact (n).
Swiss 3T3 fibroblast (30)	76.7%	43.3%
Swiss 3T3 fibroblast + STI571 (15)	13.3%	0.0%

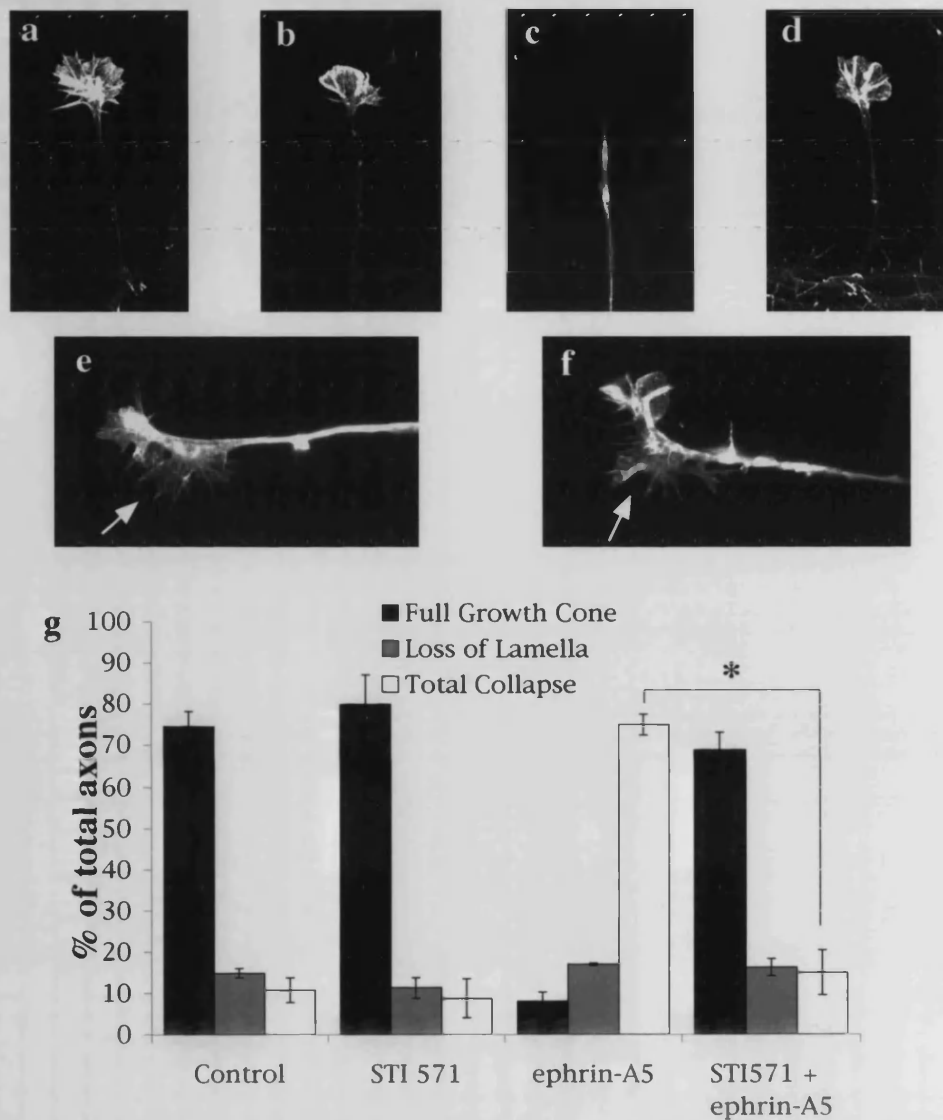
still in contact with the ephrin-A-expressing fibroblast (Fig 5.2, +15 min, between arrowheads), which, as discussed in section 5.3, might reflect an attractive response. Together the data described above demonstrate that STI571 prevents loss of RGC growth cone lamellæ and axon retraction in response to contact with an ephrin-A-expressing fibroblast, and suggest that Abl kinase is involved in mediating the ephrin-A-induced RGC repulsive response.

5.2.2. STI571 inhibits ephrin-A5-Fc-induced RGC growth cone collapse.

Since STI571 treatment of co-cultured RGCs and Swiss-3T3 fibroblasts could effect either cell type it is important to determine that the inhibition of RGC repulsive response by STI571 is cell autonomous. The effect of STI571-treatment of RGCs stimulated with ephrin-A5-Fc was therefore investigated.

Typical examples of the actin morphology of RGC growth cones treated with ephrin-A5-Fc and/or STI571 are shown in Fig 5.3. Incubation with 10µM STI571 has no effect on the actin morphology of RGC growth cones compared to control; both have spread lamellæ and filopodia (Fig 5.3a and b). The loss of growth cone lamellæ and filopodia induced by ephrin-A5-Fc (Fig 5.3c) are both prevented by incubation of RGCs with STI571 prior to stimulation with ephrin-A5 (Fig 5.3d). Figs 5.3e and 5.3f show examples of STI571- and ephrin-A5-treated RGCs that have lamellæ protruding along the axon behind the growth cone. This lamella extends along the axon for a distance of more than twice the growth cone width (Fig 5.3e and f), similar to the lamella protrusion of the RGC axon in contact with an ephrin-A-expressing

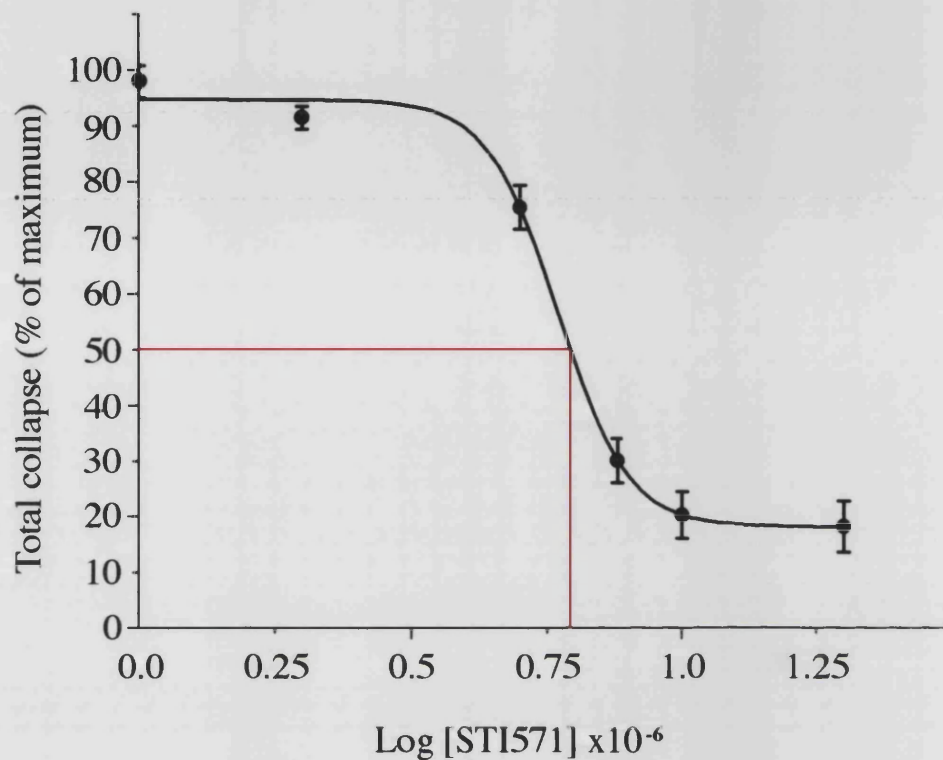
Figure 5.3. STI571 inhibits ephrin-A5-Fc-induced RGC growth cone collapse.



STI571 inhibits ephrin-A5-Fc-induced growth cone collapse of RGCs. Phalloidin staining shows that RGCs treated with anti-Fc control have full growth cones with spread lamella and filopodia (a). Treatment with STI571 (10 μ M) alone for 20 minutes has no effect on the F-actin morphology of growth cones (b). Stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes triggers loss of lamella and filopodia (c) that is inhibited by pre-treatment with STI571 for 20 minutes (d). A subset (approximately 10 %) of STI571/ephrin-A5-treated RGCs exhibit lamella protrusion along the distal portion of the axon (see arrows e, f). Quantification of STI571-dependent inhibition of ephrin-A5-induced RGC growth cone collapse shows that pre-treatment with STI571 (10 μ M) for 20 minutes significantly reduces the percentage of RGCs that lose both filopodia and lamella (Total collapse) in response to stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes (g, * $p < 0.0001$, student's T-test for 2-tailed data of unequal variance).

fibroblast shown in Fig 5.2. This morphology is rare, occurring in less than 10% of STI571-treated axons stimulated with ephrin-A5-Fc, but is never seen in RGCs treated with STI571 alone, and therefore might be indicative of an attractive response to ephrin-A (see section 5.3). Quantification of the STI571-dependent inhibition of ephrin-A5-induced growth cone collapse is shown in Fig 5.3g. Incubation with STI571 has no effect on the percentage of RGC axons with a full growth cone (control = $74.4 \pm 3.7\%$, STI571 = $79.9 \pm 7.2\%$, $p > 0.1$; Student's T-Test for 2-tailed data of unequal variance). Stimulation of RGCs in culture with ephrin-A5-Fc induces total collapse of $75.0 \pm 2.5\%$ of RGC axons, but pre-treatment of RGCs with STI571 significantly reduces the percentage of RGCs that exhibit total collapse in response to ephrin-A5 (14.9% , $p < 0.0001$; Student's T-Test for 2-tailed data of unequal variance, Fig 5.3g). To date, pharmacological inhibitors that have been reported to inhibit ephrin-A5-induced RGC growth cone collapse have only a partial effect (Cheng et al., 2003; Wahl et al., 2000). The STI571-mediated inhibition of ephrin-A5-induced RGC growth cone collapse described in this chapter is complete, as the percentage of STI571-treated axons that exhibit total collapse in response to ephrin-A5 is not significantly different from the percentage of control RGC axons without lamella and filopodia ($p > 0.2$; Student's T-Test for 2-tailed data of unequal variance). To estimate the IC_{50} of STI571 for ephrin-A5-induced RGC growth cone collapse RGCs were incubated with increasing concentrations of STI571 for 20 min prior to stimulation with $1 \mu\text{gml}^{-1}$ ephrin-A5-Fc. Fig 5.4 shows that low micromolar concentrations of STI571 have very little effect on ephrin-A5-induced growth cone collapse, and fitting the curve using MicroCal Origin provides an estimate of the IC_{50} for inhibition of collapse of $5.7 \mu\text{M}$ (with 95% confidence limits). Abl kinase activity is inhibited by STI571 *in vitro* with an IC_{50} in the sub-micromolar range, but intact cells require treatment with STI571 at concentrations between 5 and $10 \mu\text{M}$ in order to completely inhibit Abl kinase activity, as assayed by autophosphorylation (Buchdunger et al., 1996; Carroll et al., 1997; Corbin et al., 2002; Okuda et al., 2001). STI571 is routinely used at concentrations between 5 and $10 \mu\text{M}$ to inhibit Abl-dependent cytoskeletal changes in many cell types, including neuronal cells (Burton et al., 2003; Finn et al., 2003; Lu et al., 2002; Master et al., 2003). Inhibition of ephrin-A5-induced RGC growth cone collapse is complete but not saturated at

Figure 5.4. Dose-response curve of ephrin-A5-induced RGC growth cone collapse in the presence of STI571.



RGCs were treated with increasing concentrations of STI571 for 20 minutes prior to stimulation with ephrin-A5-Fc (1 μ g/ml) for 10 minutes. Ephrin-A5-induced collapse is inhibited by STI571 with an IC₅₀ of 5.7 μ M (see red line). Maximum collapse is the response induced in RGCs stimulated with 1 μ g/ml ephrin-A5-Fc in the absence of STI571.

10 μ M (Fig 5.4), therefore the use of 10 μ M STI571 in the preceding and subsequent experiments is justified.

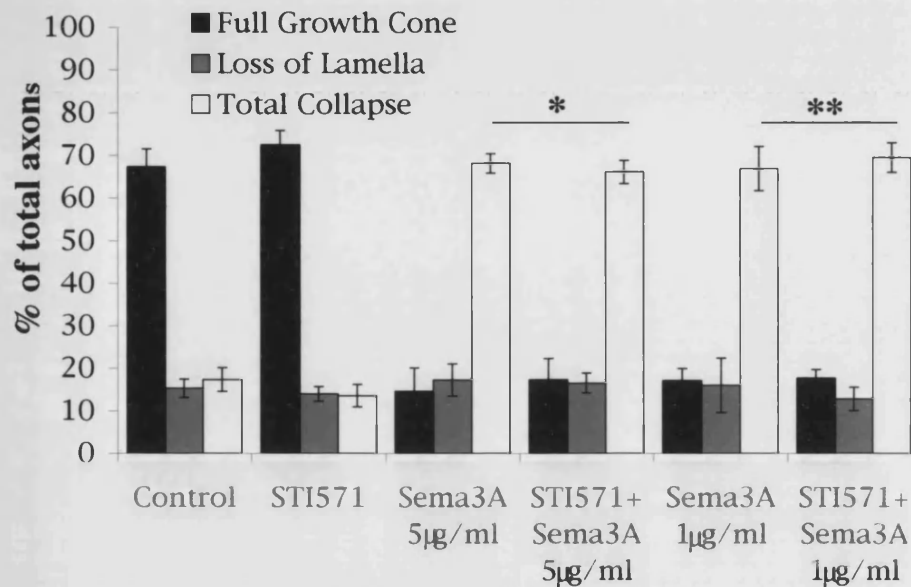
5.2.3. STI571 does not inhibit Semaphorin3A-induced growth cone collapse.

To determine whether the effects of STI571 are specific for the repulsive response induced by ephrin-A stimulation, I have investigated the effect of 10 μ M STI571 on DRG growth cone collapse induced by Semaphorin3A (Fig 5.5). Semaphorin3A induces total collapse of approximately 60-70% of DRG growth cones (68.1 \pm 2.6% in response to 5 μ gml⁻¹ Semaphorin3A, 66.9 \pm 5.2% in response to 1 μ gml⁻¹ Semaphorin3A). Treatment of DRG neurons with STI571 alone does not affect the percentage of growth cones with full lamellæ and filopodia compared with control DRG axons (Fig 5.5, full growth cone control = 67.3 \pm 4.2%, STI571 = 72.4 \pm 3.6%, $p > 0.2$; Student's T-Test for 2-tailed data of unequal variance). In contrast to ephrin-induced collapse of RGCs, STI571 does not significantly reduce the percentage of DRGs that totally collapse in response to 5 μ gml⁻¹ Semaphorin3A (Semaphorin3A = 68.1 \pm 2.6%, + STI571 = 66.1 \pm 2.7%, $p > 0.1$; ; Student's T-Test for 2-tailed data of unequal variance) or 1 μ gml⁻¹ Semaphorin3A (Semaphorin3A = 66.9 \pm 5.2%, + STI571 = 69.5 \pm 3.5%, $p > 0.1$; Student's T-Test for 2-tailed data of unequal variance). These data suggest that the effect of STI571 is specific to growth cone collapse mediated by EphA receptor signalling.

5.2.4. STI571 does not inhibit ligand stimulated Eph kinase activity.

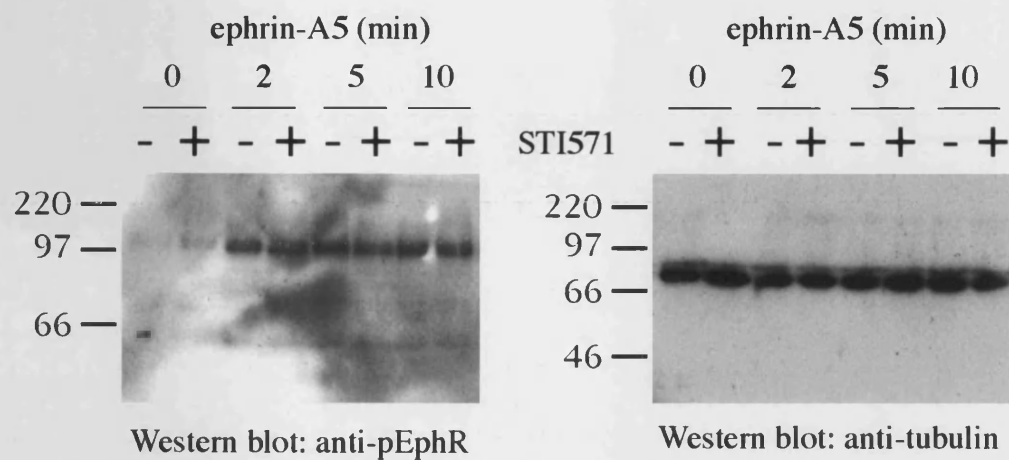
STI571 was designed as a tyrosine kinase inhibitor, and in addition to Abl has been shown to inhibit PDGF receptor, and the stem cell factor receptor c-kit, both receptor tyrosine kinases (Buchdunger et al., 2000; Carroll et al., 1997). In order to rule out any effects of STI571 on Eph kinase activity isolated RGCs were incubated with 10 μ M STI571 prior to stimulation with ephrin-A5-Fc for various times (Fig 5.6). Stimulation of RGCs with ephrin-A5 induces a rapid and sustained increase in Eph receptor phosphorylation, which is not inhibited by pre-treatment with STI571 (Fig 5.6, compare lane 3 with lane 4, and lane 5 with 6). Since this antibody specifically recognises Eph receptor phosphorylation at the conserved juxtamembrane tyrosines, which correlates with Eph kinase activity (Binns et al., 2000; Marston et al., 2003; Shamah et al., 2001), Fig 5.6 demonstrates that STI571 does not inhibit ephrin-A5-

Figure 5.5. STI571 does not inhibit Semaphorin3A-induced DRG growth cone collapse.



Treatment with STI571 (10µM) for 20 minutes has no effect on the percentage of DRG axons that lose both filopodia and lamella (Total collapse) in response to stimulation with Sema3A (1µg/ml or 5µg/ml) for 10 minutes (* $p > 0.7$, ** $p > 0.4$, student's T-test for 2-tailed data of unequal variance).

Figure 5.6. STI571 does not inhibit ephrin-A5-induced Eph receptor activation.



Isolated RGCs in culture were stimulated with 1 μ g/ml ephrin-A5-Fc for the time indicated after incubation with STI571 (10 μ M {+}) or DMSO control (-) for 20 minutes. Cells were lysed with RIPA buffer and lysates were separated by SDS-PAGE and blotted with anti-phospho-Eph receptor antibody (left panel) to monitor receptor activity. Treatment of RGCs with STI571 has no effect on ephrin-A5-induced Eph receptor phosphorylation. Cell lysates were blotted with anti-tubulin antibody (right panel) as a control for protein levels.

stimulated Eph kinase activity in RGCs and is therefore inhibiting ephrin-A-induced loss of lamella and axon retraction by some other mechanism.

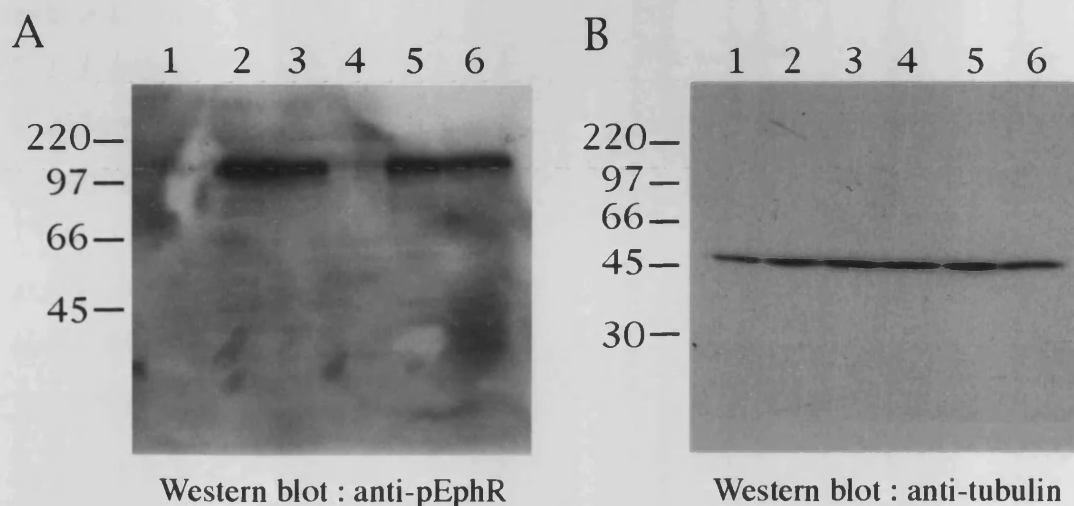
5.2.5. STI571 inhibits the association of Mena with active Eph receptors.

Since STI571 is known to inhibit the activity of Abl kinase, it is possible that Abl kinase activity is required for ephrin-A-induced growth cone collapse. Interaction between Eph receptors and Abl has been described previously (Yu et al., 2001) and therefore one possible mechanism by which ephrin-A5 stimulates growth cone collapse and axon retraction of RGCs is via EphA-mediated activation of Abl kinase activity.

The mammalian Ena/VASP family member Mena is localised to the growth cone of neurons in culture, and can be tyrosine phosphorylated by Abl (Lanier et al., 1999; Tani et al., 2003). Given the role for Ena/VASP proteins, including Mena, in the regulation of lamellæactin dynamics (Bear et al., 2002; Loureiro et al., 2002; Rottner et al., 1999a), Mena is therefore a potential link between Abl kinase activity and actin dynamics in the growth cone following EphA activation. To investigate whether Abl activity is increased following EphA activation an attempt was made to analyse the levels of phosphorylated Mena in isolated RGCs following ephrin-A5 stimulation. Unfortunately attempts to detect either total or tyrosine phosphorylated Mena in RGC lysates, whether treated or untreated with ephrin-A5-Fc, failed. It is likely that the amounts of material are limiting for this experiment. In order to address this issue therefore, I have used COS cells transiently transfected with EphA4. EphA4 is expressed by chick nasal RGCs in culture, and has been reported to interact with the Abl SH2 domain (Monschau et al., 1997; Yu et al., 2001). Expression of dominant negative EphA4 abolishes the repulsive response of chick nasal RGC axons to ephrin-A5 *in vitro*, demonstrating that this receptor is required for ephrin-A5-induced signals in these neurons (Walkenhorst et al., 2000).

COS cells transfected with empty vector (COSmock) do not exhibit detectable levels of phosphorylated Eph receptor when unstimulated, and there is no detectable increase in the level of Eph receptor phosphorylation following stimulation of these cells with 1 μgml^{-1} ephrin-A5-Fc for 10 min (Fig 5.7A lanes 1 and 4). This suggests that COSmock cells do not express significant levels of endogenous EphA receptors,

Figure 5.7. COS cells transiently transfected with EphA4 express high levels of active Eph receptor.



A. COS cells were transiently transfected with EphA4 (COSEphA4 {lanes 2,3,5,6}), or empty vector (COSmock {lanes 1,4}), and proteins expressed for 24 hours. Cells were then treated with control IgG (lanes 1-3) or stimulated with clustered ephrin-A5-Fc (1 μ g/ml {lanes 4-6}) for 2 minutes (lanes 1,2,5) or 10 minutes (lanes 3, 4, 6). The cells were lysed with RIPA buffer and lysates were separated by SDS-PAGE. Cell lysates were blotted with anti-phospho-Eph receptor antibody (A) to monitor receptor activity, or anti-tubulin antibody (B) for protein loading control. COSmock cells do not express detectable levels of phosphorylated Eph receptors following control or ephrin-A5-Fc treatment.. COSEphA4 express highly phosphorylated Eph receptors with or without ephrin-A5-Fc addition.

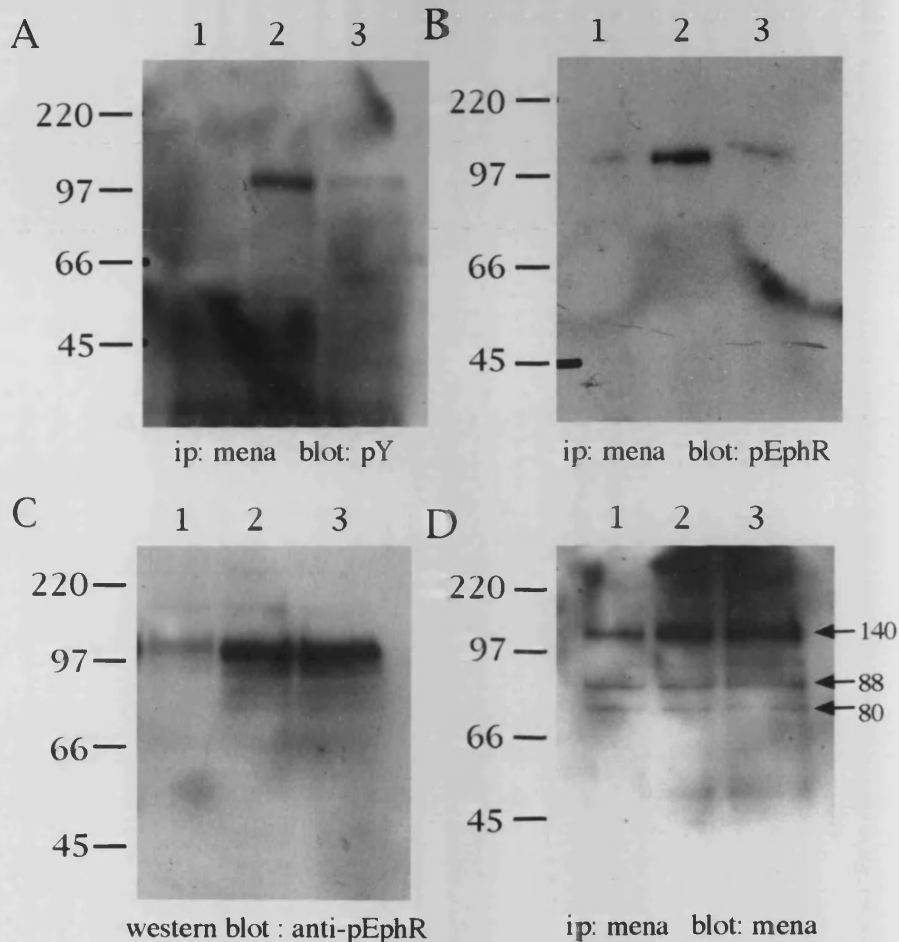
B. 1 = COSmock , 2 = COSEphA4 control 2 min, 3 = COSEphA4 control 10 min,
 C. 4 = COSmock + ephrin-A5-Fc 10 min, 5 = COSEphA4 + ephrin-A5-Fc 2 min,
 D. 6 = COSEphA4 + ephrin-A5-Fc 10 min.

making them a suitable cell line for use in this study. COS cells transfected with a plasmid encoding EphA4 (COSEphA4) exhibit high levels of phosphorylated Eph receptor compared to mock-transfected cells (Fig 5.7 lanes 2 and 3). Expression of EphA4 has previously been shown to lead to Eph autophosphorylation irrespective of ligand stimulation (Ogita et al., 2003). In agreement with this, stimulation of COSEphA4 with ephrin-A5-Fc does not lead to a detectable increase in phosphorylation of Eph receptor above unstimulated levels (Fig 5.7 lanes 5 and 6). These results demonstrate that transfection of COS cells with EphA4 results in expression of active Eph receptors, and that ligand stimulation of these cells does not further increase this Eph receptor activity. For this reason the following experiments were carried out on COSEphA4 cells in the absence of exogenous ligand stimulation.

Initial experiments were performed to determine whether endogenous Mena is phosphorylated in cells in which EphA4 is active. Abl phosphorylates Mena on a single tyrosine residue (Tani et al., 2003) but phospho-specific antibodies against Mena are not commercially available. Instead I used an anti-Mena antibody to immuno-precipitate endogenous Mena from COSmock and COSEphA4 cells, and then blotted these immuno-precipitates with an antibody against phosphorylated tyrosine residues. Fig 5.8A shows that a tyrosine-phosphorylated protein of greater than 100kD in size is present in Mena immuno-precipitates from COSEphA4 cells but not in Mena immuno-precipitates from COSmock cells (Fig 5.8A lanes 1 and 2). This tyrosine-phosphorylated protein is not present in Mena immuno-precipitates from STI571-treated COSEphA4 cells (Fig 5.8A).

Three isoforms of Mena are present *in vivo*, of molecular weights 140, 88, and 80 kd (Gertler et al., 1996); all three isoforms are expressed in COS (Fig 5.8D arrows) and therefore the phosphotyrosine-positive band seen in the Mena immuno-precipitates from COSEphA4 cells could correspond to the largest isoform of Mena, which is the only isoform tyrosine-phosphorylated *in vivo* (Gertler et al., 1996). However phosphorylated Eph receptors are also around this size (Fig 5.8C) (Marston et al., 2003; Ogita et al., 2003; Shamah et al., 2001). Initially I attempted these immuno-precipitations using a fairly high stringency lysis buffer, containing an ionic detergent and a high salt concentration, but this approach failed to yield detectable levels of Mena in COS lysates. The results shown in Fig 5.8 were obtained using a low salt lysis buffer containing a non-ionic detergent (see IP buffer, see section

Figure 5.8. STI571 disrupts the interaction between Mena and active Eph receptors in COS cells transiently transfected with EphA4.



A. COSmock (lane 1) and COSEphA4 cells with (lane 3) or without (lane 2) STI571 treatment (10 μ M, 2 hours) were lysed using IP buffer. Mena was then immunoprecipitated using an anti-Mena antibody. Mena immuno-precipitates were separated by SDS-PAGE and blotted with antibodies against phosphotyrosine (A), phospho-Eph receptor (B), or Mena (D). COSEphA4 Mena immuno-precipitates blotted for phospho-tyrosine show a single band at 110-140kD, which is inhibited by treatment with STI571. Mena immuno-precipitates show a single band at a similar molecular weight when blotted for active Eph receptor. Blotting Mena immuno-precipitates for Mena shows bands at around 140, 88 and 80kD (D, arrows). Blotting whole cell lysates (C) demonstrates that STI571-treatment does not inhibit Eph receptor phosphorylation.

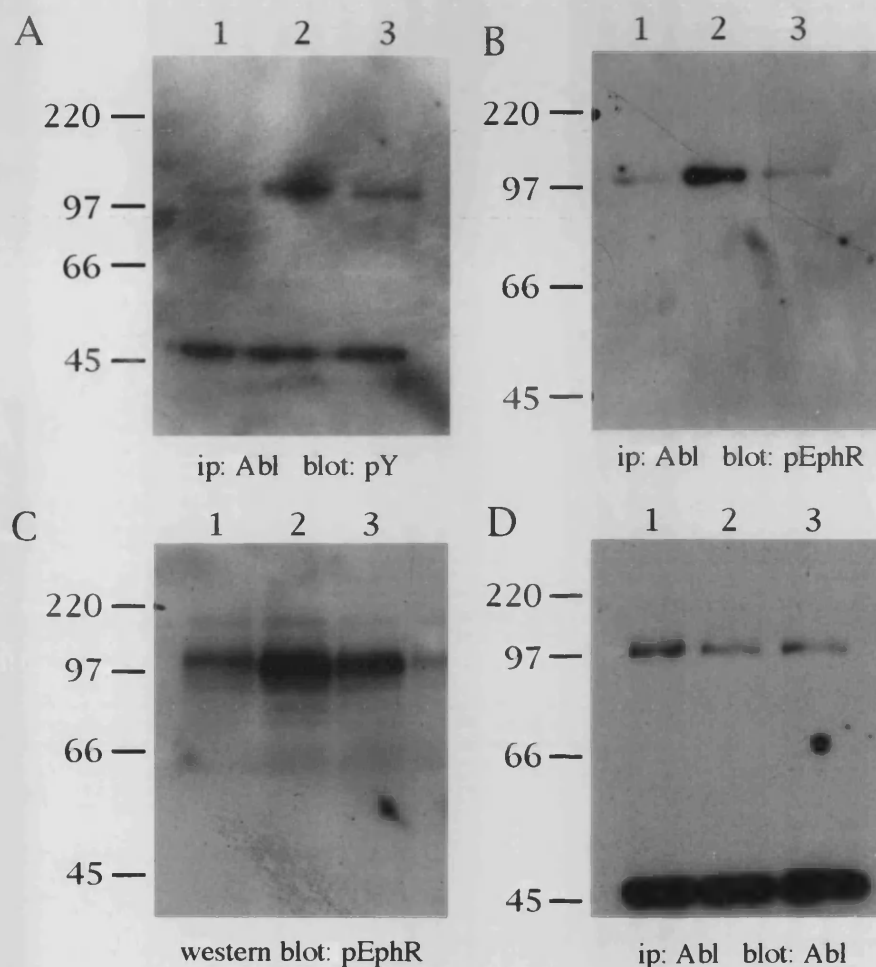
B. 1 = COSmock, 2 = COSEphA4, 3 = COSEphA4 + STI571

2.1.3). It is therefore possible that protein complexes within the cell are not disrupted under these conditions and that the phospho-tyrosine signal in Mena immunoprecipitates from COSEphA4 comes at least in part from phosphorylated Eph receptor. To investigate this possibility Mena immunoprecipitates were blotted with anti-phospho Eph receptor antibody (Fig 5.8B). Fig 5.8 shows that active Eph receptor associates with Mena in COSEphA4 cells, and that treatment of these cells with STI571 inhibits this interaction. The total level of phosphorylated Eph receptor in the cells is unchanged by incubation with STI571 (Fig 5.8C) demonstrating that treatment of COS cells with STI571 specifically disrupts the association of active Eph receptor with endogenous Mena.

5.2.6. STI571 inhibits the association of Abl with active Eph receptors.

Abl kinase activity towards exogenous substrates is stimulated by autophosphorylation at several sites, as well as direct phosphorylation of Abl's activation loop by exogenous kinase activity (Brasher and Van Etten, 2000; Tanis et al., 2003). Since Abl can associate with EphA4, and is directly phosphorylated by EphB2 *in vitro* (Yu et al., 2001), it is possible that EphA receptor activity could lead to the phosphorylation and activation of Abl kinase. To determine whether Abl phosphorylation correlates with Eph receptor activity, Abl immunoprecipitates from COSmock and COSEphA4 were blotted with an anti-phospho-tyrosine antibody (Fig 5.9A). This approach yielded poor results under a variety of experimental conditions, but a representative example of the results I was able to achieve is shown in Fig 5.9A. Abl immunoprecipitates from COSEphA4 lysates contain a tyrosine-phosphorylated protein, and the presence of this protein is inhibited by STI571 (Fig 5.9A lanes 2 and 3). This band is approximately the correct size to be phosphorylated Abl (Compare Fig 5.9A with Fig 5.9D), but again is likely to be at least in part phosphorylated Eph receptor, since blotting Abl immunoprecipitates with anti-phospho-Eph receptor antibody reveals the presence of active Eph receptor in the Abl immunoprecipitate (Fig 5.9B). Treatment of COSEphA4 cells with STI571 inhibits the association between Abl and phosphorylated Eph receptors (Fig 5.9B), although the total level of phosphorylated Eph receptor is unaffected by the presence of STI571 (Fig 5.9C, see also 5.8C). These results demonstrate that treatment of

Figure 5.9. STI571 disrupts the interaction between Abl and active Eph receptors in COS cells transiently transfected with EphA4.



COSmock (lane 1) and COSEphA4 cells with (lane 3) or without (lane 2) STI571 treatment (10 μ M, 2 hours) were lysed using IP buffer. Abl was then immuno-precipitated using an anti-Abl antibody. Abl immuno-precipitates were separated by SDS-PAGE and blotted with antibodies against phosphotyrosine (A), phospho-Eph receptor (B), or Abl (D). COSEphA4 Abl immuno-precipitates blotted for phosphotyrosine show a single band at 110-120kD, which is inhibited by treatment with STI571. Abl immuno-precipitates show a single band at a similar molecular weight when blotted for active Eph receptor. Blotting Abl immuno-precipitates for Abl shows a band at a similar molecular weight that is present in all conditions. Blotting whole cell lysates (C) demonstrates that STI571-treatment does not inhibit Eph receptor phosphorylation.

1 = COSmock, 2 = COSEphA4, 3 = COSEphA4 + STI571

COSEphA4 with STI571 abolishes the association between active Eph receptors and endogenous Abl. From the above results I cannot conclude that the phosphorylation state of Abl or Mena changes with activation of EphA receptors, since the phosphorylated protein present in Abl or Mena immuno-precipitates from COSEphA4 cells could correspond to active Eph receptors. Overall the data presented above demonstrate that incubation of COSEphA4 cells with STI571 inhibits the association between active Eph receptors and both Abl and Mena.

5.3. Discussion.

In Chapter 5 I have presented evidence that the Abl kinase inhibitor STI571 prevents the repulsive response of RGC growth cones to stimulation with ephrin-A. Treatment of RGCs with STI571 dramatically inhibits RGC loss of lamellæ and axon retraction, both in response to contact with an ephrin-A-expressing cell and to soluble ephrin-A5-Fc. This inhibition is specific to ephrin-A-induced growth cone collapse, as the same concentration of STI571 has no effect on Sema3A-induced collapse of DRG growth cones. The presence of STI571 inhibits signalling downstream of EphA receptor activation, since STI571 does not affect ephrin-A5-induced Eph receptor activation in isolated RGCs. Active Eph receptors are associated with Abl and Mena in COSEphA4 cells, and treatment of these cells with STI571 disrupts these interactions, suggesting that an Abl-Mena-Eph receptor complex is necessary for the EphA-mediated loss of RGC lamellæ and axon retraction. To my knowledge this is the first report of Mena associating with Eph receptors and the first evidence that Abl has a role in ephrin-induced cellular responses.

5.3.1. STI571 prevents association of Eph receptors with Abl and Mena.

Treatment of COSEphA4 cells with 10 μ M STI571 specifically disrupts the association of Abl and Mena with active Eph receptors, without affecting the levels of Eph receptor phosphorylation (Figs 5.8 and 5.9). Abl can adopt an inactive conformation mediated by a series of intramolecular interactions involving its SH2- and SH3- domains. The Abl SH3 domain interacts with the SH2-kinase linker region and the Abl SH2 domain interacts with the C-terminal kinase lobe, rendering the kinase inactive (Nagar et al., 2003). STI571 is thought to inhibit Abl kinase activity

by binding and stabilising the inactive conformation of Abl, since mutation of sites specifically involved in maintaining the auto-inhibited conformation confer resistance of Abl kinase to STI571 (Azam et al., 2003; Smith et al., 2003). Studies using the yeast two-hybrid system have revealed that the intracellular tail of EphA4 interacts with the Abl SH2 domain (Yu et al., 2001). STI571-mediated stabilisation of the auto-inhibited conformation, in which the Abl SH2 domain interacts with the Abl kinase domain, might preclude interaction between Abl and active EphA4. This would explain the absence of active Eph receptor in Abl immuno-precipitates from COSEphA4 cells treated with STI571. It should be noted that the results presented in Fig 5.9 do not address whether Abl is found in association with inactive Eph receptors. Full-length Abl can interact with kinase-dead EphB2 when co-transfected in 293 cells, but the association is weaker than with wild-type EphB2 (Yu et al., 2001), suggesting that the Eph-Abl interaction may at least in part be regulated by Eph receptor activity.

Fig 5.8 demonstrates that Mena also associates with active Eph receptor in COSEphA4 lysates. To date no direct interaction between any Ena/VASP family member and Eph receptors has been reported, but Abl SH3 binds Mena directly (Gertler et al., 1996), therefore it is possible that Abl mediates the interaction between Mena and active Eph receptors. Since Mena binds the Abl SH3 domain Abl could act as an adaptor, using it's SH2 domain to bind EphA4 and it's SH3 domain to bind Mena. STI571 stabilisation of the inactive conformation of Abl, in which Abl SH3 domain is occupied due to intramolecular interactions, might preclude Abl binding to Mena and therefore prevent the formation of a trimolecular complex containing Abl, Mena and active Eph receptor. This would explain the absence of active Eph receptor in Mena immuno-precipitates from EphA4 COS cells treated with STI571.

5.3.2. STI571 blocks RGC lamellæcollapse in response to ephrin-A stimulation.

The STI571-dependent disruption of the association between Abl and Mena with active Eph receptors correlates with the ability of STI571 to completely inhibit loss of RGC lamellæin response to an ephrin-A-expressing cell (Figs 5.1 and 5.2, table 5.1) and following stimulation with ephrin-A5-Fc (Fig 5.3). These results suggest

that Abl kinase activity and/or association of active Eph receptor with Abl and Mena may be required for ephrin-A-induced loss of RGC lamellæ. Recent evidence has shown that mammalian Ena/VASP family members bind to the barbed ends of actin filaments, promoting their elongation by antagonising actin capping proteins, and this is thought to underlie the direct correlation between the accumulation of Ena/VASP proteins at the very tip of the extending lamellæ and the rate of lamellæ protrusion (Bear et al., 2002; Rottner et al., 1999a). It is possible that recruiting Mena to Eph receptor-Abl complexes allows capping proteins access to the barbed ends of filaments, antagonising the role of Mena on actin filament elongation during lamellæ protrusion. STI571, by excluding Mena from the Eph receptor-Abl complex may lead to the persistent protrusion of lamellæ even in the presence of active EphA receptors. It has been shown that sequestering Ena/VASP proteins away from the membrane results in slow protrusion of persistent lamellæ, but this behaviour was investigated after stable expression of a peptide sequence that targets Ena/VASP proteins to abnormal cellular locations (Bear et al., 2002). Acute introduction of a peptide which sequesters Mena from its correct subcellular location results in rapid loss of Ptk2 cell lamellæ (Gertler et al., 1996; Southwick and Purich, 1994). Although the mechanism by which this lamella loss occurs is unknown, these observations provide evidence that rapid sequestering of Mena from F-actin in the growth cone lamella, by ephrin-A-induced association of Mena with Eph receptors, might induce RGC lamella collapse.

Alternatively it is possible that recruitment of Mena to Eph receptor-Abl complexes enhances Mena's activity on actin filament elongation by increasing Mena localisation at the membrane. Fibroblasts in which Ena/VASP proteins are sequestered at the membrane, where active Eph receptor-Abl complexes are located, exhibit rapid lamellæ protrusion and retraction, which is thought to reflect instability of the long, unbranched actin filaments (Bear et al., 2002; Krause et al., 2002). It is possible that Eph receptor activation promotes the formation of unstable actin filaments by recruiting Mena to the membrane, and that in combination with ROCK-mediated retraction (Chapter 4 and see section 5.3.3) and possible EphA-mediated loss of adhesion (see section 1.5.2), the instability of these filaments underlies ephrin-induced loss of RGC growth cone lamellæ. Since STI571 prevents Mena association with active Eph receptors, either of the above mechanisms could explain

the observations present in this chapter, that STI571 prevents ephrin-A-induced loss of RGC growth cone lamellæ.

From the results presented in this chapter, it is not clear whether Abl kinase activity is required for ephrin-A-mediated growth cone collapse. Although phosphorylation by cyclic-dependent kinases regulates Mena's association with F-actin, no information is available on the effect of Abl-mediated phosphorylation on Mena's activity in actin filament elongation. It is possible that Abl kinase activity is required for the formation of an Abl-Mena-Eph receptor complex, for example if the autophosphorylated conformation is necessary to bind to Eph receptors and/or Mena. Abl SH2 domain alone can bind to full length EphB2, suggesting that Abl activity is not required for this interaction (Yu et al., 2001). In addition it is not clear whether Mena phosphorylation is correlated with Eph receptor activation. Abl can phosphorylate Ena in the polyproline-rich region, inhibiting it's binding to Abl SH3 domain (Ahern-Djamali et al., 1999; Comer et al., 1998). Although none of the Abl phosphorylation sites in Ena are conserved in Mena, the Abl phosphorylation site does lie in the proline-rich domain of Mena (Gertler et al., 1996; Tani et al., 2003), suggesting that phosphorylation here may also disrupt binding to SH3 domain-containing ligands. It is therefore possible that the complex does not contain high levels of phosphorylated Mena, as this might antagonise Mena binding to Abl.

Expression of EphA4 in COS cells does not correlate with a shift in Mena's electrophoretic mobility (Fig 5.8D) whereas a significant change in mobility is seen after phosphorylation of Mena on two serine residues (Gertler et al., 1996; Loureiro et al., 2002). It is possible that the resolution of the gel shown in Fig 5.8D is not sufficient to reveal a detectable shift in Mena mobility following phosphorylation of a single tyrosine residue by Abl. It is likely however that the resolution of the gel in Fig 5.8A would be sufficient to expose a doublet if both tyrosine-phosphorylated Mena (approximately 140kD (Gertler et al., 1996) and phosphorylated EphA4 (approximately 110kD (Becker et al., 1995) were present in COSEphA4 lysates, suggesting that the phosphorylation state of Mena may not change in response to Eph receptor activation. Interestingly mutation of all Abl phosphorylation sites in Ena leads to only a small reduction in Ena function (Comer et al., 1998) suggesting that Abl may regulate Ena in ways other than by phosphorylation. Recent evidence from *Drosophila* suggests that Abl modulates the actin cytoskeleton by regulating Ena's

subcellular localisation (Grevengoed et al., 2003). It is therefore possible that activation of RGC EphA receptors does not change the phosphorylation state of Mena, but leads to reorganisation of the cytoskeleton by recruiting Mena to Eph receptor complexes.

5.3.3. STI571 blocks RGC axon retraction in response to ephrin-A stimulation.

The results presented in this chapter demonstrate that incubation with 10 μ M STI571 completely inhibits both the loss of lamella and axon retraction of RGCs in response to ephrin-A stimulation. I have shown in chapter 4 that axon retraction is dependent on ROCK activity, suggesting that STI571 may somehow regulate the Rho-ROCK pathway in these neurons.

The SH2/SH3-containing adaptor protein Crk is an Abl binding partner and substrate (Feller et al., 1994; Ren et al., 1994). Expression of the viral Crk homologue leads to increased Rho and ROCK activity in 293 cells, and induces the formation of contractile actin cables in neuronal cells (Altun-Gultekin et al., 1998; Iwahara et al., 2003; Tsuda et al., 2002). Recently it has been shown that Crk is required for Rho-mediated cytoskeletal rearrangements downstream of active EphA3. Ephrin-A5-Fc stimulation of EphA3-expressing cells results in the retraction of cellular processes, and this response depends on the activity of both Rho and ROCK (Lawrenson et al., 2002). Expression of SH3-mutated Crk blocks ephrin-A5-induced process retraction, and abolishes the ephrin-A5-induced increase in Rho activity in these cells (Lawrenson et al., 2002), suggesting that Rho activation is downstream of Crk. Abl can bind to Crk SH3 domain and phosphorylate a tyrosine residue close to the Crk SH3 domain (Feller et al., 1994; Ren et al., 1994). Phosphorylation of Crk on this residue allows intramolecular binding to Crk SH2 domain (Rosen et al., 1995), and therefore the traditional model has been that Abl phosphorylation negatively regulates Crk dependent signalling by preventing Crk binding to SH2 and/or SH3 binding partners. Although mutating the Abl phosphorylation site increases Crk binding to some partners, this mutation does not increase Crk dependent signalling, as would be expected if Abl phosphorylation negatively regulates Crk (Abassi and Vuori, 2002). There is evidence that translocation of Crk to the plasma membrane requires phosphorylation at the Abl site, and that this in turn is required for Crk-

mediated actin rearrangements (Abassi and Vuori, 2002), which suggests that Abl phosphorylation can positively regulate Crk-dependent signalling. In addition Crk phosphorylation has been shown to lead to a switch in binding partners (Khwaja et al., 1996), suggesting that Abl phosphorylation of Crk activates Crk-dependent signalling pathways by regulating the localisation and/or binding partners of Crk.

It is possible that EphA activation induces Abl-mediated Crk phosphorylation, and this in turn activates the Rho-ROCK pathway to induce axon retraction. How Crk expression leads to RhoA and ROCK activation is unclear. To date Crk has not been shown to interact with a Rho GEF, but the N-terminal SH3 domain of CrkII has been shown to bind the GEFs C3G and SOS (Knudsen et al., 1994; Okada and Pessin, 1996; Tanaka et al., 1994; Uemura et al., 1997), raising the possibility that an as yet unidentified Rho GEF may interact with this region of Crk. The observation that a mutation of Crk SH3 domain alone prevents ephrin-A5-induced Rho activation supports this theory (Lawrenson et al., 2002). The Rho family GEF Ephexin is found constitutively associated with EphA4, and has been implicated in ephrin-A-induced growth cone collapse (Shamah et al., 2001). This study did not investigate the mechanism by which Ephexin's GEF activity towards Rho is stimulated by EphA activation, and although to date no functional or biochemical interactions between Ephexin and Crk have been shown, it is tempting to speculate that Crk may be involved in Abl-mediated axon retraction in response to ephrin-A.

As discussed above it is not clear that STI571 prevents axon retraction via inhibiting Abl kinase activity, but does disrupt Abl and Abl substrates interacting with active Eph receptors. Interestingly Crk specifically associates with active EphA3, and there is some evidence that this interaction is mediated by another molecule (Lawrenson et al., 2002). Since Abl can bind to both Eph receptors and Crk, it is possible that Abl-Crk interaction at the site of active Eph receptors contributes to the RGC response to ephrin-A stimulation. Interestingly disrupting Abl-Crk interaction leads to loss of constitutive Rho-dependent stress fibres, and prevents serum-induced stress fibres (Nakashima et al., 1999), suggesting that interfering with Abl-Crk binding antagonises RhoA signalling. If STI571 prevents Abl-Crk interaction by imposing structural constraints on Abl this may have a similar effect on Rho-induced contractile filaments within the axon, inhibiting Rho-dependent axon retraction following EphA activation. It would be interesting to investigate whether Crk is

present in the Abl-Eph receptor complex in COS cells, and whether STI571 has any effect.

5.3.4. STI571 specifically inhibits ephrin-A-induced growth cone collapse.

Although treatment of RGCs with 10 μ M STI571 completely inhibits ephrin-A5-Fc-induced growth cone collapse (Figs 5.3 and 5.4) it has no effect on Sema3A-induced DRG growth cone collapse (Fig 5.5). These results suggest that Eph receptors and the plexinA/Neuropilin complex required to mediate Sema3A-induced growth cone collapse activate different intracellular signalling cascades. Cdk5 is an Abl substrate that is phosphorylated following ephrin-A5 stimulation of RGCs (Cheng et al., 2003; Zukerberg et al., 2000). Since introduction of a non-phosphorylatable mutant of Cdk5 inhibits ephrin-A5-induced growth cone collapse of RGCs (Cheng et al., 2003), one possible mechanism by which EphA receptor activation leads to growth cone collapse is via Abl phosphorylation of Cdk5. Sema3A-induced DRG growth cone collapse is also inhibited by the non-phosphorylatable mutant of Cdk5 however (Sasaki et al., 2002). Therefore it is unlikely that STI571 inhibits ephrin-A5-induced growth cone collapse by interfering with Abl phosphorylation of Cdk5.

Ephrin-A and Sema3A-induced growth cone collapse also share a dependence on the Rac signalling pathway. Expression of dominant negative Rac, or disruption of Rac interaction with effectors, inhibits both ephrin-A-induced RGC growth cone collapse and Sema3A-induced DRG collapse (Jin and Strittmatter, 1997; Journey et al., 2002; Vastrik et al., 1999). Abl has been reported to increase Rac activity (Burton et al., 2003), but expression of constitutively active Rac in RGCs does not cause growth cone collapse (Journey et al., 2002). Together with the observation that STI571 has no effect on Sema3A-induced growth cone collapse, this suggests that STI571 does not inhibit ephrin-A-induced growth cone collapse by inhibiting Abl-mediated Rac activation.

Ephrin-A5 stimulation of RGCs has been shown to increase Rho activity and inhibiting Rho activity has previously been reported to inhibit RGC growth cone collapse in response to soluble ephrin-A5 (Wahl et al., 2000) Although Sema3A-induced growth cone collapse of DRGs is inhibited by function blocking antibodies against the ROCK substrate CRMP (Collapsin response mediator protein), inhibition

of Rock activity with Y27632 or introduction of a dominant negative does not inhibit Sema3A-induced growth cone collapse (Arimura et al., 2000; Goshima et al., 1995). This suggests that, unlike Semaphorin4D, Sema3A-induced growth cone collapse does not involve the Rho-ROCK pathway. Since ephrin-A-induced axon retraction is mediated by ROCK (see Chapter 4) these observations further support the hypothesis that STI571 inhibits ephrin-A-induced RGC axon retraction by antagonising the Rho-ROCK pathway.

5.3.5. Eph receptor regulation of Abl kinase activity.

As discussed above I have no evidence to suggest that Abl kinase activity is regulated by Eph receptor activation, and it is possible that the Abl kinase inhibitor STI571 inhibits the repulsive response of RGC axons to ephrin-A stimulation by disrupting Abl association with active Eph receptors rather than inhibiting an ephrin-A-induced increase Abl kinase activity. It has previously been reported that ligand stimulation of EphB-expressing cells reduces the *in vitro* kinase activity of endogenous Abl (Yu et al., 2001). If STI571 is acting to prevent ephrin-A-induced lamella collapse and axon retraction via it's ability to inhibit Abl kinase activity, it would be predicted that EphA receptor activation stimulates Abl kinase activity, which is in contrast to the effect of EphB activation. However the effect of EphA activation on Abl kinase activity has not been investigated (Yu et al., 2001). Growth cone collapse induced by ephrin-B ligands is significantly slower than that induced by ephrin-As, and has a different effect on filamentous actin (Meima et al., 1997b) although to my knowledge no direct comparison has been made between the signalling pathways downstream of EphA and EphB receptors that are involved in growth cone collapse. Interestingly, while EphA receptor activation induces Crk-dependent process retraction, EphB activation has been shown to cause Crk-dependent lamella extension (Lawrenson et al., 2002; Nagashima et al., 2002). Given that Abl phosphorylation of Crk may lead to a switch in signalling pathways it is possible that EphA and EphB receptors have opposite effects on Abl activity, and thus stimulate Crk dependent repulsive/retractive responses or attractive/protrusive responses respectively.

5.3.6. STI571-induced switch to attraction.

The soluble collapse and co-culture assays used in this thesis are powerful assays with which to identify potential signalling molecules involved in the repulsive response to axon guidance cues. These assays cannot distinguish between conditions in which the repulsive response to ephrin-A stimulation is replaced by a neutral response, and conditions that may convert the repulsive response to an attractive response. There are hints from the data presented in this chapter that STI571 treatment of RGCs does not simply induce a permissive response to ephrin-A stimulation. For example RGCs extend growth cone lamellæ over the contacting fibroblast in the presence of STI571, but this is never seen in the control situation, and in the example shown in Fig 5.1 RGC growth cone lamella preferentially spreads at sites of contact with the fibroblast. In Fig 5.2 the RGC protrudes lamella along the portion of its axon in contact with the ephrin-A-expressing fibroblast, and STI571-treated RGCs extend lamellæ along the axon only after stimulation with ephrin-A5-Fc. Since stimulation of neuronal cells *in vitro* with attractive axon guidance cues induces the formation of lamellæ and filopodia (Goldberg et al., 2000; Shekarabi and Kennedy, 2002), aberrant protrusion of lamella in response to ephrin-A may be indicative of an attractive response in the presence of STI571.

A growing body of evidence now indicates that the intracellular activity of cyclic nucleotide-dependent kinases can modulate the *in vitro* response to axonal guidance cues (Song and Poo, 2001). Recently it has been shown that ephrin-A5-induced collapse of *Xenopus* neurons is inhibited by reducing PKG activity, although again the assay used cannot distinguish between a passive response and an attractive response (Mann et al., 2003). Given that PKG and/or PKA activity can modulate the neuronal response to a number of axonal guidance cues, it is tempting to speculate that Eph receptor signalling may be similarly affected.

This idea is particularly intriguing given the ability of PKA and PKG to modulate the biological activity of Ena/VASP family members. All three vertebrate members have conserved sites for PKA/PKG, and phosphorylation of these sites in Mena is required for Mena function during cell migration (Butt et al., 1994; Gertler et al., 1996; Lambrechts et al., 2000; Loureiro et al., 2002). Phosphorylation of the equivalent site in EVL and VASP prevents interaction with Abl (Howe et al., 2002; Lambrechts et al., 2000). If Mena association with Abl and Eph receptors is required for ephrin-A-

induced growth cone collapse, it would be interesting to investigate whether Abl-Mena interaction is inhibited by PKG phosphorylation of Mena. This might be a mechanism whereby PKG switches ephrin-A-induced repulsion to attraction. Since STI571 prevents Mena interaction with Abl and active Eph receptors, it would be very interesting to investigate whether STI571 can convert the RGC response to ephrin-A from repulsion to attraction, for example using the stripe assay or the growth cone turning assay.

Chapter 6. General Discussion

In this thesis I have developed a novel assay with which to investigate signalling pathways that mediate EphA receptor-dependent contact repulsion of RGCs. Axon guidance by ephrin-As *in vitro* is traditionally studied by the related phenomenon of growth cone collapse, and using the physiologically relevant co-culture assay described in this thesis, in combination with the traditional soluble collapse assay, I have identified the non-receptor tyrosine kinase Abl as key mediator of both ephrin-A-induced lamella loss and axon retraction, and have more clearly defined the function of the Rho effector ROCK in RGC responses to stimulation with ephrin-A.

Since ephrin-As are membrane-bound molecules they guide RGCs *in vivo* by contact repulsion. I have shown that in response to contact with an individual Swiss-3T3 fibroblast, which expresses endogenous ephrin-As, nasal RGCs exhibit rapid loss of growth cone lamella followed by axon retraction. These cellular responses are robust and reproducible, and can be quantified individually. Contact with a Swiss-3T3 fibroblast induces activation of Eph receptors on the RGC growth cone, and disruption of the EphA-ephrin-A interaction prevents the contact-induced loss of RGC growth cone lamellæ and axon retraction. Together with the observation that overexpression of ephrin-A5 in a cell type that does not endogenously express ephrin-A is sufficient to induce the repulsive response, these data provide strong evidence that the RGC response to contact with a Swiss-3T3 fibroblast is mediated by neuronal EphA signalling, and is therefore an appropriate assay with which to investigate signalling pathways activated by ephrin-A stimulation.

I have shown that inhibition of ROCK activity in RGCs prevents axon retraction induced by contact with an ephrin-A-expressing fibroblast, and by soluble ephrin-A5. However, ephrin-A-induced loss of RGC growth cone lamellæ, although delayed, is not prevented by inhibition of ROCK. These observations extend previously published work investigating the role of ROCK in ephrin-A-induced RGC responses, in which the authors did not quantify these cellular events separately, or describe the RGC response dynamically (Wahl et al., 2000) and the data presented in this thesis therefore demonstrate that ROCK is not the sole mediator of ephrin-A-induced RGC repulsive responses *in vitro*. The Src family kinase inhibitor PP2 does not prevent

ephrin-A-induced RGC repulsive responses, but interestingly PP3, a structural analogue of PP2 that does not inhibit Src family kinases, prevents both the RGC loss of lamellæ and axon retraction induced by membrane tethered and soluble ephrin-A, without affecting Eph receptor activity.

Finally I have shown that the Abl kinase inhibitor STI571 prevents both the ephrin-A-induced loss of RGC growth cone lamellæ and axon retraction in response to membrane-tethered and soluble ephrin-A. STI571 does not affect ligand-induced Eph receptor activation, and its effects are specific to ephrin-A-induced neuronal responses, as STI571 does not inhibit Sema3A-induced DRG growth cone collapse. STI571 prevents the association of active Eph receptors, Abl and Mena in transfected COS cells, suggesting that this complex is required for ephrin-A-induced loss of RGC growth cone lamellæ and axon retraction. It is not clear that Abl kinase activity is required to mediate ephrin-A-induced RGC loss of lamellæ and axon retraction, and it is possible that Abl has a kinase-independent role in transducing EphA-mediated signalling in RGCs.

6.1. ROCK activity mediates ephrin-A-induced RGC axon retraction, but not loss of lamellæ.

In this thesis I have reported the novel observation that inhibiting ROCK activity in RGCs specifically prevents the axon retraction normally seen in response to membrane-tethered and soluble ephrin-A. Previous studies have reported that inhibition of ROCK in RGCs inhibits the percentage of RGCs that exhibit total collapse in response to soluble ephrin-A5-Fc (Cheng et al., 2003; Wahl et al., 2000). The data presented in this thesis agree with these reports, but extend the published observations by describing the effect of inhibiting ROCK in greater detail and dynamically, to provide evidence that ROCK activity is not critically involved in loss of lamella, but it is necessary for axon retraction.

The Rho-ROCK-MLC pathway has been shown to mediate axon retraction in a variety of neuronal cells (Amano et al., 1998; Katoh et al., 1998; Kozma et al., 1997) and inhibition of ROCK reduces the incidence of RGC axon retraction induced by contact with isolated tectal cells (Thies and Davenport, 2003)]. ROCK activity can increase MLC phosphorylation directly, as well as indirectly via downregulation of

MLCP, and this in turn induces the assembly of contractile stress fibres in non-neuronal cells (Amano et al., 1996; Fukata et al., 2001; Kimura et al., 1996, Riento and Ridley, 2003). Increasing contractility in neurons is likely the mechanism by which ROCK mediates axon retraction, as it has been shown that expression of active Rho or ROCK or a MLC mutant that mimics phosphorylation by ROCK are all sufficient to induce neurite retraction *in vitro* (Amano et al., 1998; Katoh et al., 1998; Kozma et al., 1997). Genetic studies have also implicated the Rho-ROCK-MLC pathway in axon retraction *in vivo*, and provide evidence that this pathway is negatively regulated p190RhoGAP (Billuart et al., 2001). This study also provides evidence that Src activity might positively influence the Rho-ROCK-MLC pathway *in vivo* by antagonising p190RhoGAP activity (Billuart et al., 2001). *In vitro* Src and Fyn, both of which are present in RGC growth cones and can be activated by Eph receptors (Burden-Gulley and Lemmon, 1996; Maness et al., 1988; Sharfe et al., 2003; Sorge et al., 1984; Steinle et al., 2002; Takasu et al., 2002; Vindis et al., 2003), might mediate ROCK-dependent axon retraction in response to ephrin-A via LMW-PTP-induced dephosphorylation of p190RhoGAP (Bucciantini et al., 1998; Rigacci et al., 1996; Tailor et al., 1997). However I have presented data demonstrating that the Src family kinase inhibitor PP2 does not prevent ephrin-A-induced axon retraction. It has recently been reported that a higher concentration of PP2 (25 μ M) than is used in this thesis inhibits ephrin-A5-induced RGC growth cone collapse (Wong et al., 2004) but since PP2 can inhibit EphB kinase activity (Sturtz et al., 2004), it is therefore possible that the reported inhibition of ephrin-A5-induced RGC response is due to inhibition of Eph receptor activity rather than inhibition of Src family kinases. I have shown that 10 μ M PP2 does not affect ephrin-A5-induced Eph receptor activation, and at this concentration the ephrin-A-induced RGC axon retraction is significantly delayed, but not prevented. It is possible that the delay in axon retraction reflects incomplete inhibition of Src family kinase activity, but I find higher concentrations of PP2 induce RGC growth cone collapse in the absence of ephrin-A5, which makes it impossible to titrate the effects of PP2 on ephrin-A-induced axon retraction. Other studies have demonstrated that phosphorylation of Src substrates in primary neurons is completely inhibited by 10 μ M PP2 (Crossthwaite et al., 2004; Manzerra et al., 2001), and taken together with the *in vitro* evidence that Src and Fyn may antagonise the Rho-ROCK pathway by direct phosphorylation of p190RhoGAP (Brouns et al., 2001; Haskell et al., 2001; Roof et al., 1998; Wolf et

al., 2001), it seems unlikely that Src family kinases contribute to the ROCK-mediated, ephrin-A-induced RGC axon retraction.

6.2. Abl is a key regulator of ephrin-A-induced repulsive RGC responses.

The data presented in this thesis demonstrate that the Abl kinase inhibitor STI571 prevents both the loss of RGC growth cone lamellæ and axon retraction induced by ephrin-A. This is the first report of a functional link between Eph receptor signalling and Abl to date. I have also demonstrated the existence of a complex containing active Eph receptors, Abl and Mena, which is disrupted in the presence of STI571, which suggests that this complex may be required for ephrin-A-induced loss of RGC lamellæ and axon retraction.

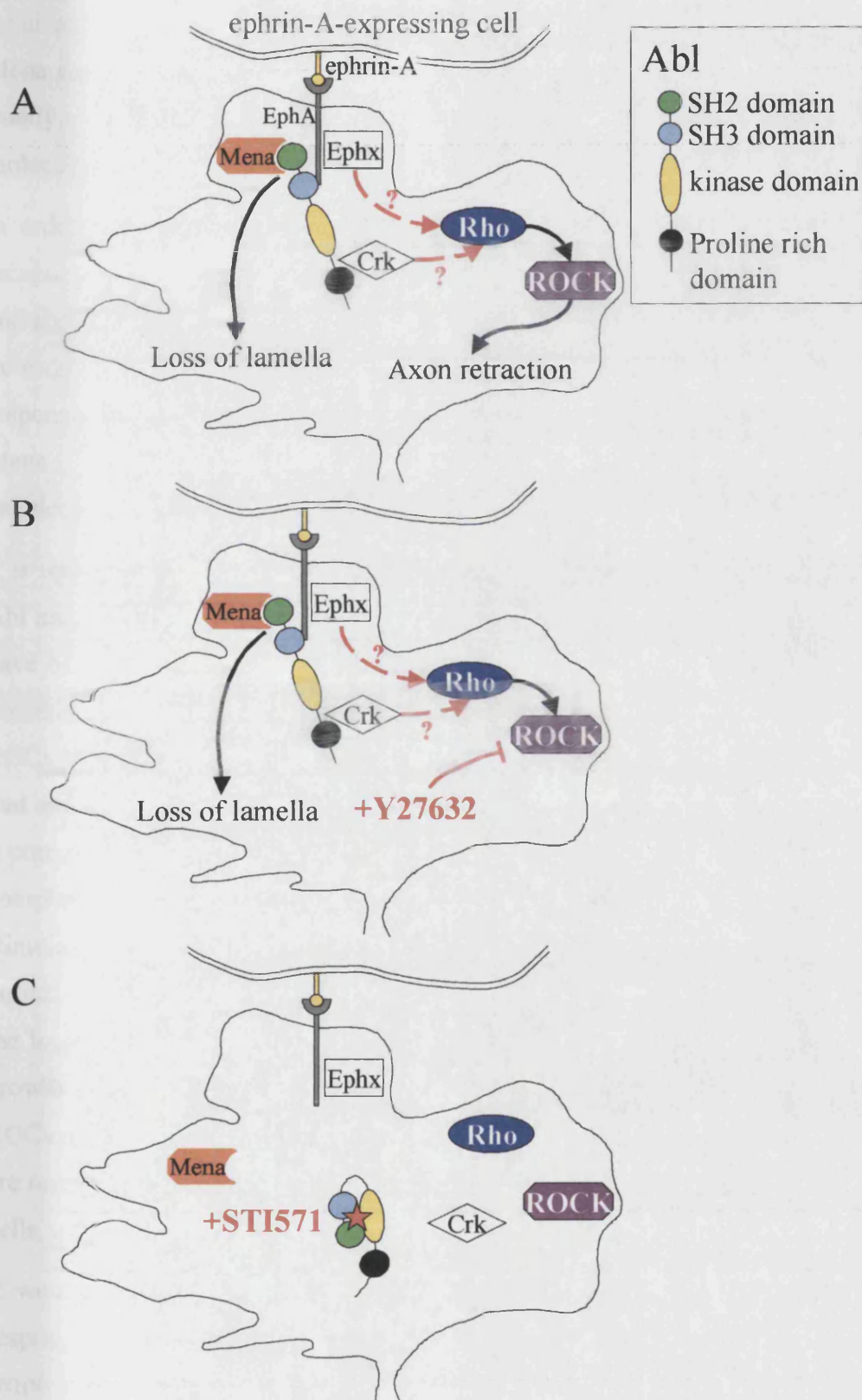
Abl has been reported to bind Eph receptors via its SH2 domain, and Mena via its SH3 domain (Gertler et al., 1996; Yu et al., 2001) and could therefore act as a scaffolding protein to assemble Eph receptor-Abl-Mena complexes. STI571 could disrupt the Eph receptor-Abl-Mena complex by stabilising the inactive conformation of Abl, in which both its SH2 and SH3 domains are involved in intramolecular interactions (Azam et al., 2003; Nagar et al., 2003; Smith et al., 2003). The ability of STI571 to inhibit ephrin-A-induced RGC loss of lamella and axon retraction correlates with its ability to disrupt the Eph receptor-Abl-Mena complex. Concentrating Mena at the plasma membrane, by recruiting it to active Eph receptor-Abl complexes, might potentiate Mena's activity towards actin filament elongation, leading to the formation of long unbranched actin filaments in the RGC growth cone lamellæ (Bear et al., 2002). The instability of these filaments may underlie the rapid loss of growth cone lamellæ in response to ephrin-A (Bear et al., 2002, Krause et al., 2002). Alternatively recruitment of Mena to active Eph receptor-Abl complexes might sequester it from its site of action at the ends of actin filaments in the growth cone lamellæ, and that this could result in rapid collapse of the lamella (Southwick and Purich, 1994). By excluding Mena from associating with active Eph receptors, STI571 could antagonise Mena-mediated loss of lamellæ in response to ephrin-A by either mechanism. From the data presented in this thesis it is not clear that Abl phosphorylation of Mena is required for ephrin-A-induced loss of RGC lamella, but it is difficult to predict whether this might contribute to Abl-mediated loss of lamella,

since to date no information is available regarding the effect of Abl-mediated Mena phosphorylation on Mena's role in actin dynamics.

STI571 also prevents ephrin-A-induced axon retraction, which I have shown to be dependent on ROCK activity. This may reflect Abl's ability to interact with Crk, which mediates both the increase in Rho activity and ROCK-dependent process retraction induced by ephrin-A5-stimulation of non-neuronal cells (Feller 1994; Lawrenson et al., 2002; Ren et al., 1994). Disruption of Abl-Crk interaction by STI571-mediated constraints on Abl conformation might be sufficient to inhibit the Rho-ROCK pathway that underlies ephrin-A-induced RGC axon retraction (Nakashima et al., 1999). STI571 is an inhibitor of Abl kinase activity, and Mena and Crk are both Abl substrates (Feller 1994; Tani et al 2003), but from the data presented in this thesis it is not clear that EphA receptor activation stimulates Abl's kinase activity. EphB activation reduces Abl activity and induces Crk-dependent lamella protrusion (Nagashima et al., 2002; Yu et al., 2001), whereas EphA-activation might increase Abl activation and induce Crk to switch binding partners to cause ROCK-mediated axon retraction (Khawaja et al., 1996; Lawrenson et al., 2002). I have discussed evidence that the effect of STI571 on Abl conformation is sufficient to prevent ephrin-A-induced loss of RGC growth cone lamella and axon retraction, suggesting that the role of Abl in ephrin-A-induced RGC repulsive responses may be kinase independent. Focal adhesion kinase FAK can act in a kinase-independent manner as a scaffolding protein to mediate assembly of a large signalling complex, setting a precedent for such a role for Abl (Carragher et al., 2003). However it would be interesting to investigate whether Abl activity, as well as phosphorylation of Mena and Crk, is increased following ephrin-A stimulation of RGCs.

The data presented in this thesis comprise the first functional demonstration of a role for mammalian Abl in transducing axon guidance signals. There is genetic evidence that D-abl is involved in mediating repulsive axon guidance cues *in vivo* (Hsouna et al., 2003; Wills et al., 2002), and therefore the data presented in this thesis, demonstrating a functional link between EphA signalling and Abl during contact-mediated RGC axon repulsion by ephrin-As *in vitro*, is an important contribution to the body of work implicating Abl in mediating axon guidance decisions.

Fig 6.1. Model for contact-induced, EphA-mediated RGC repulsive response.



For figure legend see page 180

Future Directions.

Several questions arise from the data presented in this thesis. Is Abl kinase activity required for ephrin-A-induced RGC repulsive response? Does the Eph receptor-Abl-Mena complex exist in a cell type that endogenously expresses Eph receptors? And finally, what are the effects of Abl, acting either as a kinase or as a scaffolding molecule, on RGC axon guidance *in vivo*?

In order to directly test whether Abl kinase activity changes in response to Eph receptor activation. Abl immuno-precipitated from COSEphA4 cells, in the presence and absence of STI571, could be analysed in an *in vitro* kinase assay. Related to this, in order to investigate whether the phosphorylation state of Mena changes in response to Eph receptor activation, and whether STI571 has any effect on this state, Mena immuno-precipitates from COSEphA4 cells would need to be analysed by 2D-gel electrophoresis.

It is important to verify that the complex containing phosphorylated Eph receptors, Abl and Mena, identified in COSEphA4 cells, is present in a neuronal cell type. I have been unable to immuno-precipitate Abl or Mena from RGC lysates, but it is possible that immuno-precipitation of phosphorylated Eph receptors from isolated RGCs in culture may allow the identification of such a complex in primary neurons that endogenously express Eph receptors. This approach also has the advantage that it could be used to investigate whether any members of the Eph receptor-Abl-Mena complex are constitutively associated, or whether the complex is inducible upon stimulation with ephrin-A. It would also be interesting to demonstrate the presence of an active Eph receptor-Abl-Mena complex by immunofluorescence, to investigate the localisation of the various components before and after ephrin stimulation. The growth cone collapse response to ephrin-A makes immunofluorescence studies in RGCs difficult, as the morphological structures in which these proteins are localised are rapidly lost after ephrin-A addition, but use of a neuronal cell line, such as PC12 cells, may circumvent this problem.

It would be interesting to investigate whether STI571 can convert the repulsive response of RGCs to ephrin-A into an attractive response, for example using the stripe assay, in which RGCs preferentially avoid growing on substrate-bound ephrin (Walter et al., 1987a). Since intracellular levels of cyclic nucleotides are known to

switch the response of primary neurons to a variety of axon guidance cues (Song and Poo, 2001), a related question is whether inhibiting PKG, which has previously been shown to inhibit soluble ephrin-A5-induced growth cone collapse of *Xenopus* RGCs (Mann et al., 2003), interferes with the RGC response to contact with an ephrin-A-expressing cell. It would also be very interesting to investigate the effect of PKG inhibition on the association of Abl and Mena with phosphorylated Eph receptors, as it has previously been shown that phosphorylation of other members of the Ena/VASP family interferes with their ability to bind Abl (Howe et al., 2002; Lambrechts et al., 2000).

Finally, it would be very interesting to determine whether Abl plays a role in RGC axon guidance *in vivo*. Abl and Arg double knock-out mice die early from neural tube defects (Koleske et al., 1998), therefore an alternative approach would be to inject a retrovirus encoding Abl constructs into the chick optic vesicle at early stages, and analyse the projection pattern of RGCs expressing these constructs by anterograde axonal tracing (Hornberger et al., 1999). Expression of a kinase-inactive Abl, and/or isolated Abl SH2- or SH3-domains would allow analysis of the role of Abl kinase activity and ligand binding respectively on the *in vivo* guidance of RGC axons.

Fig 6.1. Legend.

A. Under control conditions, contact with an ephrin-A-expressing cell induces formation of a protein complex including active Eph receptors, Abl and Mena. Abl acts as an adaptor in the complex, binding Eph receptors via its SH2 domain, and Mena via its SH3 domain. Recruiting Mena to this complex mediates ephrin-A-induced loss of growth cone lamella. Contact with an ephrin-A-expressing cell also triggers axon retraction, possibly via an Ephexin (Ephx) induced increase in Rho activity, and subsequent increase in ROCK-mediated actomyosin contraction, and/or possibly via Abl interaction with the adaptor Crk. **B.** The ROCK inhibitor Y27632 prevents ephrin-A-induced axon retraction, but does not affect the formation of an Eph receptor-Abl-Mena complex, and thus contact with an ephrin-A-expressing cell still induces loss of growth cone lamella. **C.** The Abl kinase inhibitor STI571 (★) stabilises Abl in a conformation in which both its SH2 and SH3 domains are occupied by intramolecular interactions, thereby preventing the formation of an Eph receptor-Abl-Mena complex (and possibly also interfering with Abl-Crk interaction) preventing both ephrin-A-induced loss of growth cone lamella, and axon retraction.

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